CYCLOSPORIN A AND RENAL FUNCTION

IN

RENAL TRANSPLANT RECIPIENTS

Gillian Eugenie Mobb

A thesis
submitted to the University of Leicester
for the Degree of

DOCTOR OF MEDICINE

1990
Chronic nephrotoxicity is the major limitation to the clinical potential of CyA in both transplantation and autoimmune disease. Retrospective investigation and a prospective study of Leicester renal allograft recipients demonstrated the excellent early graft survival, progressive deterioration of renal function and eventual graft failure characterised by this condition.

Thromboxane A₂ (TXA₂), is a powerful vasoconstrictor prostanoid which has been implicated in the mechanism of CyA-induced nephrotoxicity. Intra-renal production of TXA₂ is significantly elevated in animals and humans receiving CyA, whilst endothelial production of the vasodilator prostanoid, prostacyclin, may also be reduced. These substances were detected by radioimmunooassay of their stable hydrolysis products TXB₂ and 6-keto PGF₁α respectively, in urine and plasma.

Renal allograft function was investigated in patients receiving CyA. Isotope clearance techniques using ⁵¹Cr ethylene diamine tetra-acetic acid to measure GFR and ¹²⁵I iodohippuran to measure ERPF, were demonstrated to be superior in assessing changes in function, when compared with more commonly used parameters of serum and urinary biochemistry.

The acute effects of a single oral dose of CyA on renal haemodynamics were studied both in transplant recipients and in subjects with normal kidneys.

The response of renal function to treatment with a specific TXA₂ receptor antagonist, (GR32191B), prescribed on a double-blind placebo-controlled basis was investigated in established allograft recipients.

Renal functional reserve (RFR), believed to be prostaglandin-mediated and stimulated by protein ingestion was also investigated in these patients.

A CyA-related imbalance in prostaglandin production was clearly demonstrated. This was associated with loss of RFR capacity. TXA₂ receptor antagonism failed to ameliorate nephrotoxicity and contrary to anticipation, induced deterioration in renal function in association with a decrease in circulating concentrations of 6kPGF₁α. Possible explanations for these findings have been discussed.
ACKNOWLEDGEMENTS

I would like to express my gratitude to Professor P.R.F. Bell and Mr P.S. Veitch for their guidance and support with this work.

I would like to thank Mr. Ian Belton, MSc., MIPSM., Senior Physicist at the Leicester Royal Infirmary, for his invaluable technical assistance.

I am grateful to Dr P.M. Whiting and the staff of the Department of Clinical Biochemistry at Aberdeen Royal Infirmary, for their assistance with the prostaglandin assay.

I would like to thank Dr T. Horsburgh and the staff of the Department of Surgery laboratories at the Leicester General Hospital, for their assistance.

Advice on statistical analysis was provided by Dr Paul Burton of the Department of Community Health at the Leicester Royal Infirmary.

The figures and illustrations are the work of the Department of Medical Illustration of the Royal Gwent Hospital, Newport, to whom I am indebted.

Finally I would like to thank Mr Timothy Mobb, MA.(Classics) for reading the final manuscript.
## CONTENTS

<table>
<thead>
<tr>
<th>CONTENTS</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUMMARY</td>
<td>IX</td>
</tr>
<tr>
<td><strong>CHAPTER 1</strong></td>
<td></td>
</tr>
<tr>
<td>Evidence for the long-term deterioration of renal function in patients receiving CyA.</td>
<td></td>
</tr>
<tr>
<td>1.1 The non-renal allograft</td>
<td>1</td>
</tr>
<tr>
<td>1.2 The renal allograft</td>
<td>3</td>
</tr>
<tr>
<td>1.3 The Leicester experience</td>
<td>5</td>
</tr>
<tr>
<td>1.4 Possible causes for deterioration of renal function in a renal allograft</td>
<td>13</td>
</tr>
<tr>
<td><strong>CHAPTER 2</strong></td>
<td></td>
</tr>
<tr>
<td>Cyclosporin</td>
<td></td>
</tr>
<tr>
<td>2.1 Chemical nature and pharmacokinetics</td>
<td>19</td>
</tr>
<tr>
<td>2.2 Mechanism of action</td>
<td>20</td>
</tr>
<tr>
<td>2.3 Clinical Use</td>
<td>22</td>
</tr>
<tr>
<td>2.4 Advantages of Cyclosporin A</td>
<td>22</td>
</tr>
<tr>
<td>2.5 Side effects of Cyclosporin A</td>
<td>24</td>
</tr>
<tr>
<td>2.5.1 Acute CyA nephrotoxicity</td>
<td>28</td>
</tr>
<tr>
<td>2.5.ii Chronic CyA nephrotoxicity</td>
<td>30</td>
</tr>
<tr>
<td>2.6 Gross management of CyA nephrotoxicity</td>
<td>34</td>
</tr>
<tr>
<td>2.7 Mechanisms of CyA nephrotoxicity</td>
<td>40</td>
</tr>
<tr>
<td>2.7.i Renin-Angiotensin-Aldosterone System</td>
<td>44</td>
</tr>
<tr>
<td>2.7.ii Calcium channels</td>
<td>48</td>
</tr>
<tr>
<td>2.7.iii Sympathetic Nervous System</td>
<td>51</td>
</tr>
<tr>
<td>2.7.iv Prostaglandins</td>
<td>53</td>
</tr>
<tr>
<td>2.7.v Tubular toxicity</td>
<td>54</td>
</tr>
<tr>
<td>2.8 Prostaglandins</td>
<td>58</td>
</tr>
<tr>
<td>2.9 The effect of CyA on prostaglandin production</td>
<td>64</td>
</tr>
</tbody>
</table>
### 2.10 Theoretical approaches to modifying the CyA-induced prostaglandin imbalance

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.10.i</td>
<td>Cyclo-oxygenase inhibition</td>
<td>69</td>
</tr>
<tr>
<td>2.10.ii</td>
<td>Vasodilating prostanoids</td>
<td>70</td>
</tr>
<tr>
<td>2.10.iii</td>
<td>Fish oils</td>
<td>72</td>
</tr>
<tr>
<td>2.10.iv</td>
<td>Thromboxane synthase inhibition</td>
<td>73</td>
</tr>
<tr>
<td>2.10.v</td>
<td>Thromboxane receptor antagonist</td>
<td>75</td>
</tr>
</tbody>
</table>

### CHAPTER 3

**Methods**

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Introduction</td>
<td>78</td>
</tr>
<tr>
<td>3.2</td>
<td>Isotope measurement of renal function</td>
<td>85</td>
</tr>
<tr>
<td>3.3</td>
<td>HPLC measurement of CyA whole-blood levels</td>
<td>116</td>
</tr>
<tr>
<td>3.4</td>
<td>Radioimmunoassay of prostaglandins</td>
<td>132</td>
</tr>
<tr>
<td>3.5</td>
<td>Clinical methods</td>
<td>148</td>
</tr>
<tr>
<td>3.6</td>
<td>Patients</td>
<td>151</td>
</tr>
<tr>
<td>3.7</td>
<td>Statistical Analysis</td>
<td>154</td>
</tr>
</tbody>
</table>

### CHAPTER 4

**Results**

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Long-term trends in function in Leicester renal transplant recipients</td>
<td>155</td>
</tr>
<tr>
<td>4.2</td>
<td>Is serum creatinine a reliable indicator of renal transplant function?</td>
<td>168</td>
</tr>
<tr>
<td>4.3</td>
<td>The effect of the thromboxane receptor antagonist, GR32191B, on renal transplant function</td>
<td>155</td>
</tr>
<tr>
<td>4.3.i</td>
<td>Major adverse events</td>
<td>182</td>
</tr>
<tr>
<td>4.3.ii</td>
<td>Malignancy and Mortality</td>
<td>184</td>
</tr>
<tr>
<td>4.3.iii</td>
<td>Minor adverse events</td>
<td>185</td>
</tr>
<tr>
<td>4.3.iv</td>
<td>Transplant variables and baseline function</td>
<td>186</td>
</tr>
</tbody>
</table>
4.3.v Renal function after 3 months' treatment with placebo 190
4.3.vi Renal function after 3 months' treatment with GR32191B 194
4.3.vii Biochemistry, haematology and urinalysis 198
4.3.viii Prostanoid concentrations 200
4.3.ix Urinary biochemistry 206
4.4 The acute effect of CyA upon renal function 208
4.5 Renal Functional Reserve in patients receiving CyA 218

CHAPTER 5
Discussion 226
### APPENDICES

<table>
<thead>
<tr>
<th>I</th>
<th>Measurement of haematology, serum biochemistry and urinalysis</th>
<th>268</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>Median values of function for placebo-treated patients over the 12 month period of investigation</td>
<td>279</td>
</tr>
<tr>
<td>III</td>
<td>Placebo group renal function - raw data</td>
<td>281</td>
</tr>
<tr>
<td>IV</td>
<td>GR32191B group renal function - raw data</td>
<td>285</td>
</tr>
<tr>
<td>V</td>
<td>Patients who withdrew from the trial</td>
<td>287</td>
</tr>
<tr>
<td>VI</td>
<td>Results from GR32191B treatment groups</td>
<td>288</td>
</tr>
<tr>
<td>VII</td>
<td>Urinary prostaglandin metabolites</td>
<td>289</td>
</tr>
<tr>
<td>VIII</td>
<td>Plasma prostaglandin metabolites</td>
<td>292</td>
</tr>
<tr>
<td>IX</td>
<td>Urinary biochemistry and NAG</td>
<td>293</td>
</tr>
<tr>
<td>X</td>
<td>Data from acute CyA dosing</td>
<td>294</td>
</tr>
<tr>
<td>XI</td>
<td>Data from the investigation of Renal Functional Reserve</td>
<td>297</td>
</tr>
</tbody>
</table>

### REFERENCES

<p>| REFERENCES                                      | 298 |</p>
<table>
<thead>
<tr>
<th>CONTENTS - FIGURES</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.i</td>
<td>Annual percentage graft survival</td>
</tr>
<tr>
<td>1.ii</td>
<td>Mean annual serum creatinine concentration</td>
</tr>
<tr>
<td>1.iii</td>
<td>Reciprocal of serum creatinine v time (MK)</td>
</tr>
<tr>
<td>1.iv</td>
<td>Reciprocal of serum creatinine v time (RP)</td>
</tr>
<tr>
<td>2.i</td>
<td>Prostaglandin synthetic pathway</td>
</tr>
<tr>
<td>3.2.i</td>
<td>Portable gamma radiation detector</td>
</tr>
<tr>
<td>3.2.ii</td>
<td>Isotopes and minute timer for injection study</td>
</tr>
<tr>
<td>3.2.iii</td>
<td>Sampling venous blood</td>
</tr>
<tr>
<td>3.2.iv</td>
<td>Blood and urine samples from an infusion study</td>
</tr>
<tr>
<td>3.2.v</td>
<td>The Phillips PW4800 Automatic gamma counter</td>
</tr>
<tr>
<td>3.2.vi</td>
<td>Two compartment clearance model</td>
</tr>
<tr>
<td>3.3.i</td>
<td>Calibration chromatogram</td>
</tr>
<tr>
<td>3.3.ii</td>
<td>HPLC program</td>
</tr>
<tr>
<td>3.3.iii</td>
<td>The HPLC machine in Leicester</td>
</tr>
<tr>
<td>3.3.iv</td>
<td>Flow chart for HPLC technique</td>
</tr>
<tr>
<td>3.3.v</td>
<td>HPLC chromatogram from patient (RP)</td>
</tr>
<tr>
<td>3.4.i</td>
<td>Amersham $^{125\text{I}}$ RIA kit</td>
</tr>
<tr>
<td>3.5.i</td>
<td>Glaxo study plan</td>
</tr>
<tr>
<td>3.6.i</td>
<td>Patient undergoing isotope infusion study</td>
</tr>
<tr>
<td>4.1.i</td>
<td>Annual percentage graft survival</td>
</tr>
<tr>
<td>4.1.ii</td>
<td>Mean annual serum creatinine concentration</td>
</tr>
<tr>
<td>4.1.iii</td>
<td>Changes in median GFR and ERPF over 12 months</td>
</tr>
<tr>
<td>4.1.iv</td>
<td>Changes in median SeCr and Cl$_{Cr}$ over 12 months</td>
</tr>
<tr>
<td>4.1.v</td>
<td>Changes in median RVR over 12 months</td>
</tr>
<tr>
<td>4.1.vi</td>
<td>Changes in CyA dose and levels over 12 months</td>
</tr>
<tr>
<td>4.2.i</td>
<td>Serum creatinine v isotope GFR</td>
</tr>
<tr>
<td>4.2.ii</td>
<td>SeCr v GFR for GFR &lt; 45ml/min</td>
</tr>
<tr>
<td>Section</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>4.2.iii</td>
<td>SeCr v GFR for GFR &gt; 45ml/min</td>
</tr>
<tr>
<td>4.2.iv</td>
<td>GFR v creatinine clearance</td>
</tr>
<tr>
<td>4.2.v</td>
<td>GFR v reciprocal of serum creatinine</td>
</tr>
<tr>
<td>4.2[vi]</td>
<td>True GFR v Gault and Cockcroft &quot;GFR&quot;</td>
</tr>
<tr>
<td>4.2.vii</td>
<td>True GFR v G &amp; C &quot;GFR&quot; - 3 months later</td>
</tr>
<tr>
<td>4.2.viii</td>
<td>Change in isotope GFR v change in G &amp; C &quot;GFR&quot;</td>
</tr>
<tr>
<td>4.4.i</td>
<td>Percentage change in GFR, ERPF and CyA level (JS &amp; SMc)</td>
</tr>
<tr>
<td>4.4.ii</td>
<td>Percentage change in GFR, ERPF and CyA level (RC &amp; HW)</td>
</tr>
<tr>
<td>4.4.iii</td>
<td>Percentage change in GFR and ERPF with whole blood CyA concentration (CMc &amp; IE)</td>
</tr>
<tr>
<td>4.4.iv</td>
<td>Percentage change in GFR and ERPF with whole blood CyA concentration (GP &amp; IA)</td>
</tr>
<tr>
<td>4.5.i</td>
<td>Mean values of GFR and ERPF before and after protein</td>
</tr>
<tr>
<td>4.5.ii</td>
<td>Percentage change in GFR and ERPF after protein</td>
</tr>
<tr>
<td>4.5.iii</td>
<td>Individual changes in GFR after protein</td>
</tr>
<tr>
<td>5.i</td>
<td>Prostaglandin synthetic pathway</td>
</tr>
</tbody>
</table>
SUMMARY

Cyclosporin A (CyA) was introduced into clinical practice in 1982 as a specific immunosuppressive agent. Since then a marked improvement in 1 year renal allograft survival has been demonstrated. In the days of conventional therapy with azathioprine and steroids, 1 year graft survival rarely exceeded 60%. Following the introduction of CyA, this figure has increased to between 80 and 90% in most centres. After several years of use, however, it became apparent that these excellent, early results were not maintained and that a gradual, progressive, deterioration occurred in the number of grafts continuing to work at end of each subsequent year. To date, large studies suggest an annual graft loss of between 5 and 6% (Evans 1989), whilst a projected figure of only 25% survival for renal allografts reaching 10 years has been quoted by Terasaki (1989).

An explanation for this progressive deterioration of kidney function has been widely sought by both animal and clinical investigation. It has become clear that in addition to an early, acute nephrotoxic, reversible effect, long term treatment with CyA, even in low dose regimens, produces a progressive chronic nephrotoxicity.

Good evidence exists for the production of an intense intra-renal vasoconstriction resulting from CyA treatment. It is this vasoconstriction which is thought to be the primary, physiological mechanism responsible for the nephrotoxicity of this drug.

A variety of chemical mediators of this effect have been proposed and there exists experimental evidence from in vitro and in vivo studies to support each suggested mechanism.
This work concentrates its attention upon the intra-renal production of prostaglandins, which are themselves intimately involved in the maintenance of renal function, particularly in the presence of renal dysfunction. Increased renal production and excretion of the vasoconstrictory prostanoid thromboxane A$_2$ (TXA$_2$) has been clearly demonstrated in patients taking CyA, whilst in tissue culture and certain animal models, CyA appears also to inhibit the production of the vasodilatatory prostanoid prostacyclin (PGI$_2$).

Enzyme inhibitors have been used experimentally, to reduce the production of certain relevant prostaglandins and hence correct the CyA-induced prostanoid imbalance. However, none of these agents has produced a complete reversal of CyA-induced changes in animal models of renal dysfunction.

In this study, a specific thromboxane receptor antagonist, (GR32191B - Glaxo Group Research) has been given to a group of established renal allograft recipients in an attempt to reverse the nephrotoxic side effects of CyA. In addition to this, the acute effect of CyA therapy was studied in a group of patients being treated for the skin condition psoriasis and its longer term effect was investigated in a group of renal transplant patients who had not received GR32191B.

A study was also performed to investigate Renal Functional Reserve. Normal kidneys and those of patients receiving azathioprine immunosuppressive therapy retain this capacity which can be utilised in times of deteriorating function. As functional reserve is thought to be mediated by prostaglandins, it would be interesting to know if it were present in patients receiving CyA immunosuppressive therapy.
CHAPTER 1
EVIDENCE FOR THE LONG TERM DETERIORATION OF RENAL FUNCTION IN PATIENTS RECEIVING CyA THERAPY

1.1 The Non-renal Allograft

Depression of renal function by CyA has been clearly demonstrated in heart transplant recipients by Tomlanovich et al (1986) and Myers et al (1988). Wheatley et al (1987) and Gonwa et al (1988) have demonstrated a similar deterioration in liver transplant recipients with previously normal renal function. A recently published study described the effect of an intravenous infusion of 4mg/kg CyA given to 8 healthy female volunteers. A significant depression of Glomerular Filtration Rate (GFR) occurred within 3 hours although the decrease demonstrated in Effective Renal Plasma Flow (ERPF) did not reach statistical significance (Weir et al 1989).

Much debate exists as to whether long term therapy with CyA produces stable or steadily deteriorating renal function. Porter and Bennett (1986) suggested that the native kidney might be more susceptible to the toxic effects of CyA whilst the effect on a transplanted kidney is less severe. In heart transplant recipients with previously normal renal function, a steady decline has been demonstrated, with 50% loss of GFR after 1 year of CyA therapy and a further 18% loss by the end of the second year (Tomlanovich et al 1986, Myers et al 1984). In 1988, Myers et al reported that 5 out of 73 heart transplant recipients in their unit, had developed end stage renal failure (ESRF) requiring dialysis or renal transplantation, despite maintenance of a good cardiac output. In this paper it was also shown that in a proportion of these patients, reducing or even stopping CyA therapy did not always arrest the progressive renal dysfunction. Similar results were also reported by
Greenberg and co-workers (1987) who used only the measurement of serum creatinine to assess function in heart transplant recipients. The percentage of patients with normal function ascertained by a normal serum creatinine level, was 55% at 6 months. This figure had decreased to zero by 36 months, whilst again reducing the CyA dose did not produce any marked improvement.

Non-transplant patients receiving CyA for autoimmune disease also demonstrate a progressive deterioration of renal function. Nahman et al (1987) reported two such cases. Both patients developed severe progressive renal functional impairment after only a few months of treatment. Stopping CyA led to a return of serum creatinine to a normal value in one, whilst the other suffered an irreversible deterioration of renal function.

Not all studies, however, demonstrate the same findings. Accurate isotopic measurements of GFR performed by Gonwa et al (1988), demonstrated early deterioration yet no progressive loss of filtration capacity from 6 weeks to 2 years post-operatively in the native kidneys of liver transplant recipients.

Stable long term measurements of serum creatinine have been reported by both de Francisco and co-workers (1987) in pancreatic transplantation and Kahan (1986) in heart transplant patients from Houston, Texas. However, in studies where serum creatinine levels remain constant it is possible that discrete, deteriorating trends in true GFR and ERPF are not being detected by this less sensitive parameter.

In conclusion, from the available literature it would seem that in many instances, native healthy kidneys are susceptible to the progressive nephrotoxic effects of CyA. Some authors believe the renal allograft to
be more sensitive to the effects of potential toxins (Land 1987) and therefore demonstrates more definite dysfunctional changes following the introduction of CyA immunotherapy.

1.2 The Renal Allograft

Since the first experimental use of CyA in a small study of renal transplant recipients by Calne and his team in 1978, the nephrotoxicity of CyA has been appreciated. In this early study, large initial doses of 25mg/kg were given and some of the toxic effect demonstrated has since been diminished by reducing the size of the initial loading and subsequent maintenance doses. However, despite a widespread trend towards dose reduction, the nephrotoxic effect of CyA remains, regardless of the size of dose. Significant impairment in inulin and para-amino-hippuric acid (PAH) clearances was demonstrated by renal transplant recipients given initial doses as low as 10mg/kg (Hauser et al 1988) and maintenance doses of 5mg/kg (Vanrenterghem et al 1985).

As the initial functional capacity of a cadaveric renal allograft remains a mystery, both the immediate and long term effects of CyA are even more difficult to interpret. However, there are now many reported "conversion" studies in which marked improvement in renal transplant function occurs following cessation of CyA and introduction of azathioprine immunosuppression (Morris et al 1983 [Oxford], Thistlethwaite et al 1985 [Chicago], Hall et al 1987 [Australia]). In addition to experimental animal work, these studies suggest that the nephrotoxicity which results from short term CyA administration therapy may have a large functional component and is therefore reversible. However, conversion studies in patients with previously normal renal function do not consistently demonstrate a return of functional parameters to the baseline level after stopping CyA. For example,
Hamilton et al (1982) described the conversion of 5 hepatic transplant recipients treated with CyA: 4 showed an improvement in renal function, however, in only 1 patient did serum creatinine concentration actually return to the normal range. Hunt et al (1987) described 15 heart transplant recipients in 14 of whom, function improved after conversion but again neither serum creatinine nor creatinine clearance ($C_{\text{Cr}}$) returned to baseline, pre-transplant levels. This was, however, after more than 12 months therapy with CyA.

Many authors unwisely use serum creatinine (SeCr) levels as their only means of monitoring renal function and have consequently reported no significant long term deterioration in renal transplant function (Scolari et al 1987, Merion et al 1984). A source of possible error in the interpretation of this and other similar data may result from the widespread use of SeCr to assess renal function. Many authors refer to this or its inverse as a reliable indicator of glomerular filtration rate. However, SeCr may not rise above the normal range until GFR has fallen by 50% or more (Feehally et al 1983, Tomlanovich et al 1986). Therefore, a stable normal SeCr level does not automatically correlate with a steady normal GFR or RBF. Curtis et al (1986b) demonstrated this in a conversion study of renal transplant recipients, in whom changing to azathioprine therapy produced a significant decrease in mean arterial pressure (MAP) and renal vascular resistance (RVR); a significant increase in ERPF but no significant change in SeCr level was observed. Plotting SeCr against GFR demonstrates an exponential relationship which can be transformed to linear by plotting the $\log_{10}$ or inverse of SeCr concentration. In renal transplant patients taking CyA, all of whom demonstrate a degree of dysfunction, a marked variation in individual values of SeCr for a given GFR occur. Consequently, it would be unwise
to infer a change in GFR from parallel changes in SeCr concentration or alternatively, stability of renal function when SeCr levels remain similar. The only reliable way of following trends in renal function is to perform regular isotope measurements of GFR and ERPF.

Short term follow up in patients with an uncomplicated post-operative course is perhaps less likely to demonstrate gradual loss of renal function. Both Post et al (1987) and Wilkinson et al (1987) measured GFR and found non-significant decreases over an 11 or 12 month period. However, renal transplant patients followed for longer periods clearly demonstrate a gradual deterioration of function. Vanreentghem et al (1987) demonstrated gradually climbing SeCr concentrations during the second post-transplant year. Once SeCr starts to climb beyond the normal range, an appreciable amount of function has already been lost. Similar trends in SeCr were noted by Flechner et al (1983) whose patients also showed progressive deterioration.

In conclusion, it is felt that all renal allograft recipients demonstrate a degree of renal dysfunction which results from treatment with CyA. However, this depression of function and its subsequent progression may not be detectable by the relatively insensitive methods used by most units to monitor renal function. Consequently, reports of "normal" early and "stable" longer term function need to be interpreted with caution. Only when accurate estimations of GFR and ERPF are widely performed will the true picture emerge.

1.3 The Leicester experience
CyA was introduced into the Leicester Renal Transplant Unit immunosuppressive regimen in mid 1983. Since that time, 1 year graft survival rates have reflected results published by other centres,
FIG. 1.1 Percentage graft survival per year following transplantation.
exceeding 70% as demonstrated in Figure 1.i. However, from this graph it can also be seen that annual graft survival then follows a steady, progressive decline over successive years. This graft loss seems to occur at a regular rate as described by other CyA users. It is also apparent that no deceleration occurs in the rate of graft loss, even as late as the fourth or fifth year after transplantation.

Until 1988, graft function was assessed in Leicester by regular monitoring of SeCr concentrations. On reviewing those patients treated with CyA whose grafts were still functioning towards the end of 1989, (Fig 1.ii), again a progressive and relentless deterioration in renal function is demonstrated by rising SeCr concentrations. This progressive dysfunction has occurred despite regular monitoring of CyA whole blood trough levels by High Performance Liquid Chromatography (HPLC) and appropriate dose adjustment to maintain therapeutic concentrations.

Temporal changes in renal function can be illustrated by plotting the inverse of SeCr concentration (1/SeCr) against time. The individual plots of two patients from Leicester are shown in Fig (1.iii) and (1.iv) Patient MK was transplanted in 1984 and patient RP in 1985. Both graphs demonstrate CyA-related relentless deterioration in function, with both grafts failing before the end of the fifth year.

In addition to these progressive functional changes another trend is also seen in the overall Leicester renal transplant population. Whilst transplant function clearly deteriorates with time, the level of function demonstrated at the end of the first year appears to have shown a progressive improvement from 1984 to 1986. However, in the following years, early graft function became more disappointing.(Fig 1.ii)
FIG. 1.ii Mean serum creatinine concentration per year following transplantation, for kidneys still functioning in 1989.
Kidney failed 6 months later

FIG. l.iii Reciprocal of serum creatinine v time in a patient transplanted in 1984. (MK)
FIG. 1.1v Reciprocal of serum creatinine v time in a patient transplanted in 1985. (RP)
Patients transplanted in 1986, however, not only displayed the best SeCr concentrations at the end of the first year but also demonstrated better 1 year survival when compared with most other years.

It is possible that the progressive improvement in early renal transplant function from 1984 to 1986 represents a "learning curve" as experience was gained in the handling and monitoring of patients receiving a new pharmacological agent. Those patients who received a transplant in 1986 had better early function and have also maintained this advantage to date. These patients also demonstrate more stable percentage graft survival with fewer grafts lost over the subsequent years.

Despite widespread education and the media drawing attention to the plight of people requiring renal transplantation, the availability of suitable organs has not paralleled an ever increasing demand. Consequently in Leicester, it has been necessary to relax the criteria for suitable donor organs. In particular, since 1986, kidneys from older donors and occasionally from well controlled diabetics have been accepted for transplantation. This is demonstrated by the relatively older median donor age in 1987 and 1988 compared with previously, and in the larger proportion of donors arising from the over 40 age group (Table 1.i).

In addition, 1989 saw the introduction of an intensive programme of living-related kidney donation to supplement the demand for donor organs.

Therefore, patients in the Leicester renal transplant unit demonstrate the changes associated with CyA therapy also seen in larger populations of similarly immunosuppressed patients. In addition, these data also
demonstrate a further cause of deterioration, which has resulted from the availability of fewer suitable donors. Clearly, urgent investigation and assessment of ways to prevent or decrease CyA nephrotoxicity in these patients is required.

<table>
<thead>
<tr>
<th>YEAR OF Tx</th>
<th>MEDIAN DONOR AGE</th>
<th>% DONORS &gt; 40YRS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1984</td>
<td>32</td>
<td>35.6</td>
</tr>
<tr>
<td>1985</td>
<td>28.5</td>
<td>38.9</td>
</tr>
<tr>
<td>1986</td>
<td>30</td>
<td>33.3</td>
</tr>
<tr>
<td>1987</td>
<td>39</td>
<td>42.2</td>
</tr>
<tr>
<td>1988</td>
<td>41.5</td>
<td>55.6</td>
</tr>
</tbody>
</table>

Table 1.1 Trends in donor age during CyA use in Leicester.

This study investigated a group of renal transplant recipients who were between 6 weeks and 2 years post-transplantation at the time of recruitment. Consequently, all patients investigated were transplanted between January 1986 and December 1988. Accurate measurements of renal function were performed using isotope injection techniques and these were compared with concomitant SeCr and Cl\textsubscript{Cr} concentrations. A group of these patients was treated with a specific thromboxane receptor antagonist in an attempt to reverse the vasoconstrictive effect and hence nephrotoxicity of CyA. Those patients who received placebo only, had repeated assessments performed over a 12 month period in order to examine functional trends demonstrated by patients transplanted during different years.
1.4 Possible causes for deterioration of renal function in a renal allograft.

Gradual deterioration of function in a renal allograft can result from combinations of a variety of factors.

(i) Intrinsic disease
Prior to harvesting, donor kidney function is routinely monitored by measurements of serum biochemistry, creatinine and hourly urine output. Any medical history of diabetes, hypertension or other potential damaging disease should be available from previous notes or relatives. However, a small risk still exists of transplanting an organ with pre-existing disease which could subsequently progress to renal failure within the recipient.

(ii) Recipient environment
A proportion of renal transplant recipients suffer renal failure as a result of a disease which has produced gradual renal damage and progressive functional deterioration. Despite careful control and treatment of hyperglycaemia and blood pressure, patients with both diabetes melitus and essential hypertension can suffer renal failure in both native and subsequently transplanted organs. Glomerular diseases in which autoimmune antibodies are formed against certain renal antigens can recur within the donor organ and contribute to progressive functional deterioration and eventual graft failure.

(iii) Transplantation
Physiological
Kidneys transplanted from living related donors demonstrate the best graft survival rates (Migliori et al 1987). One of the reasons for this is the absence of any comparable period of cold ischaemia; in addition,
periods of warm ischaemia are also reduced to a minimum. Despite the infusion of cold preserving fluid at cadaver organ harvesting, many kidneys suffer a degree of ischaemic damage which leads to an initial period of delayed function in a relatively large proportion of cases, histological features of acute tubular necrosis being demonstrated by transplant biopsies. In the majority of patients this condition recovers completely although a small number progress to primary non-function. It was generally felt that this initial delay did not affect long term function, (Ito et al 1987), however, increased susceptibility to CyA may result.

**Technical**

Renal vascular stenosis can follow renal transplantation, occurring at the site of a vascular anastomosis. This condition leads to progressive tissue ischaemia, producing irreversible chronic histological changes with subsequent deterioration in function. Surgical relief of the stenosis will produce some improvement in function although a degree of permanent loss may result.

Similarly, gradual ureteric obstruction can result from an ureteric stenosis. This commonly occurs at the site of the uretero-neocystostomy but can occur at any site along the ureter which may have suffered ischaemia as a result of vascular stripping at the time of organ harvesting. A slowly developing hydronephrosis may be asymptomatic, being detected only at a late stage by a progressive deterioration in renal function with rising SeCr concentrations. Ultra-sound scans of the graft, as are regularly performed in Leicester, avoid the late diagnosis of this complication.
**Immunological**

Progressive immunological damage suffered by a transplanted kidney is difficult both to detect and quantitate. Of necessity the majority of renal transplants are performed across an HLA mismatch of variable degree. However, strong cross match reactions are accepted indicators of the need to avoid transplantation in certain donor-recipient situations. Where cross matching results are acceptable, transplantation proceeds with the additional support of immunosuppressive therapy usually in the form of CyA or azathioprine and steroids. The aim of immunosuppression is to inhibit the formation of antibodies against the mismatched HLA antigens and also the many other antigens which are not included in modern matching techniques.

**Acute cellular rejection.**

Acute rejection episodes are usually detectable as rapidly rising SeCr concentrations suggesting an acute renal dysfunction. This is usually, but not consistently associated with tenderness over the site of the graft, pyrexia and reduced urine output. Since the introduction of CyA, clinical symptoms have become less obvious making rapid diagnosis more difficult. Confirmation of diagnosis is by renal transplant biopsy which demonstrates a characteristic monocellular infiltrate in addition to tissue oedema and vascular thromboses. Treatment is by additional pulsed steroid therapy or one of the antilymphocyte agents available. Readjustment of immunosuppressive therapy may be required after the episode has responded to acute treatment. If the episode resolves, graft function often returns to pre-rejection levels as ascertained by SeCr concentrations. However, in some instances a sustained loss of graft function can result.
Chronic rejection

Long term deterioration of renal function is thought to result from chronic cellular rejection. This condition stems from the immunological mismatch between donor and recipient stimulating continuous "low grade" antibody formation and resultant graft damage. In the pre-CyA era, this condition was characterised by loss of glomeruli, thickening of basement membranes, obliterative arteriopathy and the presence of a chronic inflammatory cell infiltrate. Once the first post-transplant year had passed with its high graft loss, the number of subsequent annual graft failures from chronic rejection was relatively low when compared with later losses in the CyA era.

(iv) Drugs

The transplanted kidney is perhaps even more susceptible to nephrotoxic agents than native organs. The periods of relative ischaemia prior to and at the time of transplantation may render the tissues more sensitive, whilst the combination of one or more nephrotoxic agents may be additive. CyA is nephrotoxic in its own right and can accentuate the toxicity of other drugs such as the amino-glycosides or trimethoprim, by interfering with metabolism or clearance and producing permanent renal damage.

Cyclosporin A

Acute CyA nephrotoxicity

Acute nephrotoxicity produced by this immunosuppressive agent appears to be a condition which is detected by a process of exclusion and which usually responds to dose reduction. The condition usually presents as an episode of acute renal dysfunction, which is not associated with the clinical and histological features of acute cellular rejection and which occurs in the absence of urinary infection or ureteric obstruction.
There are no specific histological changes which characterise this condition. In the majority of patients, circulating trough levels of CyA are elevated as detected by Radioimmunoassay (RIA) or HPLC. Renal function usually improves once the CyA dose is decreased. Renal function usually returns to baseline levels although occasionally permanent impairment of function can result.

**Chronic CyA nephrotoxicity**

This condition is even more difficult to characterise as there are no specific features to detect. Certain "characteristic" histological features have been described, including striped interstitial fibrosis and obliteratorative arteriolopathy, neither of which, however, are specific for the condition (Mihatsch et al 1988). A degree of chronic rejection is probably included in the changes attributed to this condition, as some histological features are shared by both. However, the rate of renal deterioration and progression to renal failure in some patients taking CyA is greatly increased when compared with patients on conventional azathioprine therapy.

Individual rates of functional deterioration differ between patients. This can be explained by the fact that each renal allograft suffers a different degree of intrinsic damage depending upon the ischaemic time, number of rejections, use of nephrotoxic drugs etc. and therefore the susceptibility of an individual organ to the nephrotoxic effect of CyA will vary.

Chronic renal ischaemia results from prolonged intra-renal vasoconstriction induced by CyA treatment, eventually damaging all functioning tissue. As vasoconstriction is a physiological response, an element of the measurable deterioration in renal function may be
reversible if CyA therapy is stopped. This has been demonstrated in the many drug conversion studies. While function improves, however, the risk of acute rejection is greatly increased (Veitch et al 1987).

Structural changes which result from this chronic ischaemia are, however, irreversible. The amount of available functioning renal tissue is slowly reduced, to a point at which progression to renal failure becomes inevitable despite stopping CyA therapy (Myers et al 1988).

The majority of patients probably retain a reversible element of renal function for some time. This has been demonstrated by Hunt and co-workers (1987) who performed a conversion trial 1 year after starting CyA therapy. More recently unpublished work with nifedipine has demonstrated a reversible element of renal function in patients who had received a renal allograft over 3 years previously (McNally et al. unpublished observations).

There is good evidence to suggest that the vasoactive prostanoid TXA₂ may play an important role in CyA-induced vasoconstriction. It was therefore proposed that antagonism of TXA₂ activity at its receptor might improve renal function by releasing the reversible element of renal function described. Patients who had received their renal allograft up to 2 years previously were included in the study. It was hoped, therefore, that with the use of a TXA₂ receptor antagonist, the beneficial effects of CyA could be retained without the threat of accelerated, progressive dysfunction and inevitable renal destruction demonstrated in the majority of patients receiving this agent.
CHAPTER 2
CYCLOSPORIN

2.1 Chemical nature and pharmacokinetics

Cyclosporin (Cy) was first isolated from the soil fungi, *Tolypocladium Inflatum Gams* and *Cylindrocarpum Lucidum Booth* and subsequently purified at Basel in 1973. It is a fungal metabolite with a narrow spectrum of antifungal activity.

CyA has powerful immunosuppressive qualities without the side effect of myelotoxicity. The earliest human clinical trials were performed in Cambridge, UK in 1978. In 1981 it was synthesised by Wenger which led to the subsequent production of a family of cyclosporins. Only cyclosporins A, C and G have immunosuppressive activity.

CyA is a polypeptide consisting of 11 amino acids including an unique amino acid in position 1. The compound has the chemical structure: $\text{C}_62\text{H}_{111}\text{N}_{11}\text{O}_{12}$ and a molecular weight of 1206.6 Daltons. Nine different cyclosporins have been synthesised and are identified by the suffix A to I.

The CyA molecule is strongly hydrophobic and needs to be administered orally in an oil vehicle. Approximately 40% of an oral dose is absorbed via the lymphatic system of the terminal ileum, passing in the portal circulation to the liver where the first-pass mechanism eliminates about 25% of that absorbed. Bioavailability is therefore low at around 25% (Kahan 1985).

After an oral dose the plasma concentration peaks between 2 and 4 hours. Elimination occurs in two phases, one with a half life of 2 to 4 hours and one with a longer half life of 10 to 12 hours. This necessitates twice daily dosing to maintain adequate immunosuppressive trough levels.
but may lead to intermittently high peak levels which may be dangerous.

CyA is cleared by the liver, being metabolised by the hepatic cytochrome 
P450 mono-oxygenase system and excreted by the liver into the bile.
Hence any degree of hepatic dysfunction can prolong clearance and raise 
blood levels. The great majority of metabolised drug leaves the body in 
the faeces.

As a result of this mechanism of degradation within the liver, it is 
clear that CyA will interact with other drugs which affect the same 
enzyme system. For example, ketoconazole, an imidazole antifungal 
agent, blocks enzyme activity and CyA metabolism, increasing blood CyA 
concentrations, enhancing CyA toxicity. Phenytoin and the barbiturates 
induce the enzymes responsible for CyA metabolism, tending to reduce CyA 
blood levels. Drugs which are nephrotoxic in their own right produce a 
cumulative toxicity in combination with CyA further jeopardising renal 
function. This group includes the amino-glycosides and trimethoprim. 
Non-steroidal anti-inflammatory drugs (NSAIDs) enhance CyA toxicity by 
their effect on intra-renal prostanoid production; this will be 
discussed in detail later.

2.2 Mechanism of action
Treatment with CyA induces relatively specific immunosuppression which 
appears to be selective for donor antigens. For example, peripheral 
blood lymphocytes taken from a CyA-treated pig with a heart transplant 
exhibit unresponsiveness to donor antigens in mixed lymphocyte culture 
(MLC) but not to third party antigens. CyA must be given prior to 
exposure to the foreign antigens in order to establish its 
immunosuppressive effect (Nelson 1984).

There appears to be no direct effect on B-lymphocytes nor on the T-cell
independent mechanisms of B-cell antibody production. However, T-cell dependent activities are affected by CyA.

In man immunosuppression is maintained for as long as adequate blood levels of CyA are present.

In the presence of CyA, interleukin-2 (IL-2)-producing T helper cells become unresponsive to interleukin-1 (IL-1) released by antigen presenting macrophages. IL-2 production is also inhibited and this effect seems to coincide with a blocking or reduced production of messenger RNA for this lymphokine. Finally, IL-2 receptor production may also be reduced in some systems, whilst other systems "respond" to exogenous IL-2 indicating reduced IL-2 production only (Bunjes et al 1981).

Most studies show a decrease in T helper cells with preserved numbers of T suppressor/cytotoxic cells. The activity of cytotoxic T-cells is inhibited at doses of CyA which do not affect suppressor cells.

More recently it has been noted that there is a graded dose-response of the immune cells to CyA. At a mean inhibitory concentration in 50% of systems, sub-therapeutic concentrations of CyA inhibit IL-2 production but not IL-2 dependent cell growth as demonstrated by adding exogenous IL-2. At this concentration also, induction of cytotoxic T-cells can still occur. At concentrations which equate with therapeutic blood levels, the effect of exogenous IL-2 on cytotoxic T-cells is blocked ie. they become less responsive to IL-2 at increasing doses of CyA. At similar doses, IL-2 receptor expression is also reduced. However, even at very high levels in excess of 1000ng/ml, T suppressor cell activation is still relatively resistant to CyA (Hess 1989).
In conclusion, therefore, CyA inhibits both the production and release of IL-2 from T helper cells. This in turn leads to inactivation and loss of proliferation of specifically T cytotoxic cells but allows activation of T suppressor cells (Thomson et al 1984).

2.3 Clinical Use

The major and most widespread use of CyA is in organ transplantation. It has greatly improved the results of all types of allograft but especially so in heart-lung transplantation which seemed virtually impossible with azathioprine therapy. In bone marrow transplantation, CyA has also greatly reduced the incidence of devastating graft versus host disease.

In clinical trials, CyA has been used extensively in a variety of conditions characterised collectively by the presence of immune-mediated inflammation. It has produced impressive improvements in psoriasis, Type I diabetes mellitus, uveitis, Behcet's disease, systemic lupus erythematosus and autoimmune glomerulonephritis, whilst investigation continues in rheumatoid arthritis, myasthenia gravis and multiple other conditions. However, the major drawback with CyA, especially in these non-life-threatening conditions is its proven nephrotoxicity.

2.4 Advantages of Cyclosporin A

Azathioprine was the major immunosuppressive agent used since the early years of renal transplantation. It is a purine analogue and acts within the cell nucleus, interrupting DNA replication after its own rapid metabolism to 6-mercaptopurine. It is a non-specific inhibitor of all rapidly dividing cells, most notably those within the bone marrow. The number and activity of circulating killer cells and monocytes is reduced whilst mitosis of immunologically competent lymphoid cells is inhibited
via blocking of both DNA and mRNA synthesis. However, generalised bone marrow suppression also results leading to a higher risk of opportunistic infection which can rapidly become systemic and may prove fatal.

CyA is a more specific immunosuppressive agent which has produced improvement in graft and patient survival in uncomplicated renal transplantation. 1 year survival rates increased from 60 to 80% (Canadian Multi Centre Transplant Study 1983) whilst further studies showed that at 3 years, improved graft survival was maintained compared with previous results; 66% v 42% (Calne and Wood 1985) and 70% v 60% (Opelz 1989).

In addition, infections with opportunists, in particular, bacteria and fungi, are less frequent. There does, however, appear to be less resistance to Pneumocystis carinii and Cytomegalovirus infections which occur more commonly than in the general population.

The incidence of malignancy is higher in immunocompromised patients as demonstrated by patients with the Acquired Immune Deficiency syndrome. This also applies to patients on immunosuppressive therapy. Lymphomas and leukaemias are particularly common, with a reported incidence of between 2 and 16% and a mean value of 4%. Cancers of the skin, lip and squamous cancer of the cervix appear to be more commonly seen in this group of patients whilst the solid tumours of breast, colon and prostate seem to occur with less frequency. This latter finding may be only partly explained by the mean younger age of transplant recipients (Hanto and Simmons 1986). However, despite the increased risk of malignancy, the incidence in patients on CyA therapy is lower when compared with those receiving azathioprine. Interestingly, lymphomata which occur whilst taking CyA often completely regress on cessation of therapy.
2.5 Side effects of Cyclosporin A

CyA has many side effects which are unpleasant for the patient but are not serious or life-threatening. The neuroectodermal system is often affected with many patients developing a tremor especially during the early days of high dosing. Burning and paraesthesiae, particularly in the extremities are symptoms also commonly encountered. Skin changes and coarsening of facial features have been reported and are particularly marked in children taking CyA for more than 6 months or in large doses (Reznik et al 1987). Gingival hyperplasia and soreness may require surgical trimming. Hypertricosis is a common and embarrassing problem for female patients taking CyA.

Metabolic abnormalities including hyperkalaemia, hyperchloeraemic acidosis and fluid retention have been reported (Miach 1986).

**Hypertension**

Hypertension frequently develops in renal transplant recipients taking CyA, even following native nephrectomy. Chapman et al (1987) quoted a figure of 46% at 3 months but the incidence tends to increase with time from transplantation. In another review, 80% of heart transplant recipients had acquired hypertension by the second year (Myers et al 1988).

**Hepatotoxicity**

More serious side effects which can be life-threatening or jeopardise graft function include hepatotoxicity, a thromboembolic tendency and nephrotoxicity. Fortunately in renal transplant patients hepatic toxicity is relatively rare. It is heralded by elevated concentrations of liver enzymes and bilirubin and usually responds to a reduction of
dosage or a change of therapy.

**Thrombotic tendency**

There is good evidence for a thromboembolic tendency in patients receiving CyA although some studies provide conflicting results. Brunkwall et al (1987) compared the incidence of deep vein thrombosis (DVT) in the first three post-operative weeks in a group of renal allograft recipients taking CyA with a control group receiving azathioprine. A higher incidence of DVT was recorded in the azathioprine group but was attributed to the much higher steroid doses prescribed to these patients rather than the immunosuppressive drug used. Most other work, however, supports the presence of a thrombotic tendency. In 1985, Vanrenterghem et al studied 90 patients taking CyA and 90 receiving azathioprine in the early post-transplant period. 17 thromboembolic complications, (including DVT and pulmonary embolus) occurred in 13 patients taking CyA. Only a single case of superficial thrombophlebitis was documented in the azathioprine group. It was concluded that all renal transplant recipients should receive prophylactic anticoagulation.

Coagulation studies strongly support the presence of increased coagulability as a result of CyA therapy. Thrombocytopenia and increased uptake of $^{111}$Indium labelled platelets has been demonstrated within the transplanted kidney of patients taking CyA (Schlanger et al 1986). At the microscopic level CyA appears to induce endothelial disruption which in turn encourages the deposition of platelets and fibrin leading to glomerular and afferent arteriolar thrombosis. An haemolytic-uraemic type syndrome with haemolysis and fragmentation of red cells can also occur (Neild et al 1985). It has been suggested that enhanced platelet aggregation within the kidney might release local
TXA₂, whilst systemic concentrations remain within normal limits in these patients (Benigni et al 1988b). In addition, reduction in PGI₂ synthesis may also contribute to this tendency (Neild et al 1985, Vanrenterghem et al 1985).

Nephrotoxicity
The major drawback with CyA is its well documented nephrotoxicity, which presents a problem in all patients and adds further complication to the understanding, diagnosis and management of renal dysfunction in the patient with a renal allograft.

When CyA was first used experimentally in animals, no nephrotoxicity was demonstrated at the doses required to produce immunosuppression. Therefore, when nephrotoxicity became apparent in man it presented a problem which had not previously been anticipated.

Much experimental and clinical investigation has been performed in attempting to discover the mechanism of this condition. However, it is possible that CyA nephrotoxicity is multifactorial in origin and to date no single agent has been able to reverse totally the adverse effects of this drug.

In the renal allograft recipient in particular, several factors can contribute to an overall picture of renal dysfunction. This has already been discussed on page 13.

Both an acute and a chronic form of CyA nephrotoxicity are recognised. The two conditions are distinguishable by their time of onset, speed with which renal dysfunction develops and response to treatment. An episode of acute toxicity can, however, be superimposed upon chronic graft failure. The two conditions may also be closely interrelated as
it is felt that early events which lead to renal dysfunction may increase the long term risk of developing chronic CyA damage: "long term susceptibility to the nephrotoxic effects of CyA is occasioned by events which occur in the peri-operative or immediate post-operative period" (Klintmalm et al 1984).

In order to investigate CyA nephrotoxicity thoroughly, an animal model has been sought from which the effects of CyA in man can be extrapolated. The rat is the most widely investigated species, however, different strains vary in their sensitivity to the toxic effects of the drug, whilst all require at least 3 to 4 times the human dose to produce comparable toxic effects. These larger doses produce anorexia and weight loss which can alter renal fluid and salt handling. In experimental studies paired feeding is essential to control for these effects. The responses to CyA demonstrated in the rat may differ widely from those seen in man and thus experimental evidence must be interpreted with care.

Renal cell and tissue cultures are in vitro models used to investigate the effects of CyA. Endothelial cells harvested from the human umbilical vein are also widely used and readily available for culture. Useful information has been obtained from the latter system but it must be remembered that umbilical endothelium may not always "react" in the same manner as adult vascular endothelium and as with any isolated tissue system, is not under the constraints imposed upon an organ enclosed within an intact animal (Jaffe 1984). Similarly it must be appreciated that information obtained from isolated perfused kidneys, renal slices, glomerular and renal cell culture may also not reflect in vivo findings.

In the rat, a single, large dose of CyA can produce changes suggestive
of the acute nephrotoxicity seen in man. Finding an animal model of chronic nephrotoxicity has proved more difficult. It is thought that similar doses given for longer than a week lead to the changes associated with chronic CyA nephrotoxicity and such experiments are described as using "chronic dosing".

2.5.1 Acute Cyclosporin A nephrotoxicity

In patients treated with CyA, the condition of acute nephrotoxicity can occur at any time but is more commonly seen in the early weeks or months after transplantation. It is more easily recognised clinically than the chronic type of toxicity but similarly has no characteristic specific histological features in man, despite some interesting results from work performed in animals.

The clinical condition is characterised by a fairly rapid deterioration in renal function with rising serum creatinine, potassium and urea concentrations. Other conditions which could produce a similar picture need to be excluded. This includes ureteric obstruction, generalised or local infection and acute graft rejection which requires a needle biopsy for diagnosis. In most cases there is a tendency for CyA blood levels to be elevated but this is not necessarily a consistent feature. The diagnosis is made by a process of exclusion and once made, treatment involves a rapid reduction of CyA dosage. If the deterioration of function and diagnosis are quickly recognised, the condition is usually reversible. Whilst most patients respond to a decrease in CyA dosage, the occasional patient may be hypersensitive to CyA requiring triple therapy with lower CyA doses than during routine maintenance treatment. Occasionally, conversion to conventional treatment with azathioprine is necessary.
Much has been written about the histological changes accompanying acute CyA nephrotoxicity. No recognisable specific changes have been described and all the features reported can be seen in association with other conditions found in a renal allograft. Most commonly reported from work in rats is the presence of large vacuoles, increased numbers of lysosomes, myeloid bodies and lipid droplets within the cells of the straight segment of the descending limb of the proximal convoluted tubule (Whiting et al 1982, English et al 1987). Some workers have noted the presence of giant mitochondria within the same cells but these are not specific to this condition and were not seen by Verani et al (1984) who investigated a large group of patients with acute renal dysfunction secondary to acute CyA toxicity. Acute cellular rejection can be associated with a lymphocytic infiltrate, interstitial oedema, haemorrhages and a mononuclear vessel wall infiltration, all features which can also occur with acute CyA nephrotoxicity. Verani et al (1984) noted that the mononuclear cellular infiltrate was less marked in acute CyA toxicity but that coincidence of the two conditions should always be considered.

Vascular changes have also been noted in acute CyA toxicity with intracapillary glomerular thromboses, arteriolar sclerosis, intimal thickening and mucoid fibrosis (Myers 1986). It is thought that the changes seen occur as a result of an episode of intense intra-renal vasoconstriction which can result from a single dose of CyA. Obviously in a patient with acute CyA toxicity, these effects have not followed one single dose but an additive effect of several weeks or months of therapy. However, in single dose studies in rats this phenomenon can be easily demonstrated. Acute dosing of rats produces a rapid fall in renal blood flow without a change in systemic arterial pressure and
therefore a direct increase in renal vascular resistance occurs (Sullivan et al 1985a, 1985b, Paller et al 1985). This has also been demonstrated clearly in an isolated perfused rat kidney model (Luke et al 1987). From experimental animal work and studies in patients taking CyA it appears that the renal vasoconstrictor response to CyA is probably the single most important factor in its nephrotoxicity. Patients undergoing an acute nephrotoxic episode invariably respond to reduction of CyA dosage by the return of their serum creatinine levels to previous values. Some patients may have creatinines within the normal accepted range, however, as will be demonstrated later, glomerular filtration rate and renal blood flow can be markedly impaired and renal vascular resistance similarly elevated before serum creatinine rises above the accepted "normal range" (Ross et al 1987).

2.5.ii Chronic Cyclosporin A nephrotoxicity

This condition presents a more complex problem of recognition, diagnosis and management. Myers (1986) attempted to define CyA-induced chronic nephrotoxicity as a rising serum creatinine more than 1 year after transplantation. However, in the renal transplant situation this finding must be viewed in the light of the many other possible causes of a rising serum creatinine concentration. The deterioration in renal function resulting from this condition alone seems to be more insidious, more progressive and less reversible than in its acute counterpart. It is not always easily recognised in the early stages and once the serum creatinine has started to rise above a "normal range", an appreciable amount of renal function may well be permanently lost (Ross et al 1987).

Many workers have now demonstrated the significant fall in Renal Blood Flow (RBF) and GFR with associated rise in RVR and serum creatinine seen in renal transplant patients on long term CyA (Bertani et al 1987,

Certain clinical changes are also noted with long term CyA therapy. In addition to the functional haemodynamic effects, CyA is thought by many to be a renal tubular toxin, affecting the renal handling of water and electrolytes. A metabolic hyperkalaemic acidosis may occur which is thought to be secondary to reduced secretion of H⁺ and K⁺ in the voltage-dependent segment of the distal tubule (Ryffel and Mihatsch 1986). The reduced secretion of these cations into the urine has also been demonstrated in animals (Tonnesen et al 1983). Proximal tubular handling of sodium and hence water also seems to be affected with an overall reduction in excretion and consequent sodium retention (Whiting and Simpson 1988). These findings will be discussed more fully later.

Hypertension is another common finding in patients on long term CyA therapy. In renal disease, essential hypertension can be the primary mediator of ESRF and can therefore continue to affect a transplanted organ. It is difficult to calculate the incidence of CyA-induced hypertension in these patients. Moss et al (1985), however, recorded an incidence of 80% following heart transplant and a figure as high as 60% after bone marrow transplant in patients taking CyA, with apparently normal kidneys. They suggested that lower incidences recorded in patients with renal allografts were related to the denervated state of the transplanted kidney. Myers et al (1988) reported an 85% incidence in heart transplant recipients 2 years post-operatively and this figure was still increasing with time since the operation. In these particular patients, progressively rising blood pressure may be secondary to progressive renal damage.
In animal models, a single acute dose of CyA leads to a prompt rise in blood pressure (BP) and heart rate but this change is only transient (Moss et al 1985). However, after only one week of CyA therapy, spontaneously hypertensive rats develop an early and significant elevation of BP when compared with untreated control animals. These animals normally suffer labile hypertension from 6 to 12 weeks of age and then develop persistently elevated BP. It seems that CyA therapy accelerates the development of hypertension in these animals, possibly by increasing vessel sensitivity to other vasoconstrictor agents (Nahman et al 1988, Lustig et al 1987). This model might represent one mechanism of CyA nephrotoxicity in man.

There are no specific histological features of chronic CyA nephrotoxicity. Again tubular epithelial vacuolation and giant mitochondria have been reported after 12 months of therapy (Mihatsch et al 1983). A finding documented more commonly after long term treatment with CyA is the presence of striped interstitial fibrosis and arteriolopathy. Interstitial fibrosis can occur as part of a healing process following tissue damage and is noted in many renal conditions. However, it has been documented in association with CyA therapy both in renal allografts and in the native, previously normal kidneys of patients receiving CyA for other conditions. For example both arteriolar lesions and interstitial fibrosis were seen in 6 patients who received CyA for between 4 and 36 months, for the treatment of Behcet's disease (Svenson et al 1986). Patients receiving CyA for corneal grafts also developed interstitial fibrosis and medial hypertrophy but some of these renal changes could be secondary to the very high blood pressure which occurred as a consequence of treatment (Nahman et al 1988). One interesting feature of the interstitial fibrosis found in association
with CyA therapy is its characteristic striped appearance - the
descriptive phrase often used is "tabby cat fibrosis" (Mihatsch et al
1989).

The arteriolar changes have been intensively studied by Mihatsch and co­
workers (1983, 1989) who also attempted to explain their pathogenesis.
In addition to its vasoconstrictive effects, CyA also affects vascular
endothelial integrity. The endothelium becomes more susceptible to
damage and the normally continuous processes of thrombosis and
fibrinolysis are disrupted. Consequently fibrin and platelet thrombi
are deposited on the vascular endothelium and its basement membrane
within glomeruli and arterioles. These deposits enter the vessel wall
and lead to a necrotising arteriolopathy with single cell necrosis of
both endothelium and underlying smooth muscle. Cellular regeneration
occurs covering the deposits and leading to vascular occlusion and
irreversible structural changes. These sub-endothelial deposits have
also been reported from animal studies (Bertani et al 1987).

In addition to these changes, focal glomerular sclerosis and tubular
atrophy have also been reported in association with chronic CyA
toxicity. Tubular atrophy can be seen as early as 2 months after the
onset of treatment, (Bohman et al 1985, Mihatsch 1989), whilst both
features were found in the Stanford heart transplant patients who
required dialysis as a result of CyA therapy (Myers et al 1988).

Therefore many histological changes have been documented in chronic CyA
nephrotoxicity; all can be seen in other conditions characterised by
damage to renal vessels, tubules and the interstitium. Perhaps the
striped nature of the interstitial fibrosis alone may prove to be a
diagnostic aid to this condition.
Acute CyA toxicity seems to be more characterised by functional changes which are reversible on modification of therapy. Do reversible changes occur in the presence of chronic CyA nephrotoxicity?

In an animal model of chronic CyA nephrotoxicity, Simpson et al (1988) noted that Sprague Dawley rats demonstrated irreversible histological changes after only 28 days of low dose therapy (10-20mg/kg/d). In contrast, Bertani et al (1987) found that tubular changes, including vacuolation and loss of brush border almost completely disappeared two months after stopping therapy with 40mg/kg/d. Interstitial fibrosis, however, persisted as a long term feature. This larger dose was given for 3 months in a model which perhaps more closely resembles the chronic toxicity demonstrated in man. Fibrosis, arteriolar changes and glomerular sclerosis are features which once present do not disappear on cessation of therapy. Therefore, once these changes appear a definite and irreversible loss of renal function has occurred. It could be postulated that once CyA chronic nephrotoxicity has been recognised by a rise in serum creatinine concentration, a number of previously functioning nephrons has been irreversibly damaged. Any therapeutic measures instituted may halt or slow the progressive nature of this condition but will not bring back function already lost.

2.6 Gross management of CyA nephrotoxicity

Several approaches have been used when attempting to resolve the problem of chronic CyA nephrotoxicity. Firstly, attempts have been made to modify the dosage regimens of presently available therapies to reduce the nephrotoxic effects of long term treatment with CyA. Secondly, from the results of experimental models of this condition, other drugs have been introduced in conjunction with CyA to counteract the basic mechanism or mechanisms which may be responsible for inducing renal
dysfunction.

2.6.1 Lowering CyA dose

When CyA was first included in immunosuppressive regimens, 25mg/kg/day was assumed to be a suitable loading dose. This was quickly recognised as excessive and toxic; loading doses were then reduced to 10mg/kg which in turn was found to be inadequate to prevent acute rejection. Eventually 17mg/kg was selected as an adequate starting dose and has been used in most centres for several years until more recently safe reduction to 14mg/kg/d has been introduced (Evans 1989).

Over the first 100 days relatively high doses of immunosuppression are required, which can subsequently be steadily reduced to a maintenance oral dose which according to the manufacturers' specification should fall between 4 and 6 mg/kg/day. However, in clinical practice, once a "stable" situation is reached with steady serum creatinine concentrations, CyA levels within a quoted therapeutic range and a clinically well patient, there is reluctance to reduce doses further for fear of allowing an acute rejection episode to occur. However, Evans (1989) recently calculated an annual graft loss of 6% in patients taking CyA, while Thiel (1989) reported that 8% of people with therapeutic CyA blood levels demonstrate toxicity by developing chronic CyA arteriolopathy. More accurate measurements of renal function using radio-labelled isotopes may indicate that even apparently stable patients are slowly losing function. Consequently it was suggested that halving the dose regardless of blood CyA levels, might stop this progression (Thiel 1989).

In Glasgow, Briggs (1989) has instituted low dose therapy which has brought about a decrease in serum creatinine levels. At one year
patients are taking 10mg/d prednisolone and only 3mg/kg/d CyA. After this time some patients were converted to azathioprine with poor results whilst the rest were well maintained on 10mg/d prednisolone and a mean CyA dose of 2.7mg/kg/d with no increase in the incidence of acute rejection. Similarly Van der Werf and Serota (1988) treated patients with 3 mg/kg i.v. pre-operatively, 1.0 to 1.5 mg/kg i.v. post-operatively and then between 3 and 4.5 mg/kg/d orally with 10 mg/d prednisolone. Of 106 patients in this study, 1 year survival was between 80 and 90%. However, 26 treatable rejections occurred (25%) in which one graft was lost. 9 patients required conversion to azathioprine for toxicity even at this low CyA dose. Finally, over the 1 year period, serum creatinines were not seen to alter significantly from the relatively high mean concentration of 141mmol/l at one month and 146mmol/l at 12 months. Lowering the dose of CyA seems to produce an improvement in serum creatinine concentration but the values quoted do not revert to the normal range (Levy et al 1988, Pallardo et al 1988). In addition, if lowering CyA dosage increases the risk of acute cellular rejection, this in itself can increase the sensitivity of a renal allograft to chronic CyA nephrotoxicity.

2.6.ii Triple therapy
The combination of a third immunosuppressive agent with CyA and steroids enables the CyA dose to be reduced, improving renal function, whilst avoiding the risk of graft rejection (Jones et al 1988, Miller et al 1986). However, including azathioprine in any combination drug regimen, adds the disadvantages associated with this drug. Thus an increased risk of opportunist infection and myelosuppression present extra problems for both clinician and patient.

Fries et al (1988) followed patients on triple therapy regimen of CyA,
azathioprine and steroids for a 3 year period. Serum creatinine levels remained superior in the control group who received only azathioprine and prednisolone. No progressive dysfunction was claimed after 2 years, even measuring GFR with isotopes, however, after 3 years, those receiving CyA had much higher SeCr concentrations. Interestingly, there was also more interstitial fibrosis and tubular atrophy detected on biopsy in those receiving CyA. In a similar study, Scharek et al (1987) also noted that triple therapy markedly reduced the incidence of delayed renal allograft function from 43% in patients taking CyA alone to 27% and in addition the number of acute rejection episodes was also decreased by a third. Therefore, the reduced doses of CyA given in combination with other drugs afford some but not complete protection from its nephrotoxic effects. After longer term treatment, chronic CyA nephrotoxicity is probably not avoidable by this method of management. In some centres triple therapy regimens are only commenced when a patient has previously developed dysfunction or toxicity on higher dose CyA therapy. The long term results of function in these patients may be difficult to interpret as any beneficial effect of the triple therapy may be counterbalanced by irreversible impairment of renal function which has resulted from previous dysfunctional episodes. Secondly, when given in combination with azathioprine and steroids, some centres still give relatively high doses of CyA - at least within or even above that recommended by the manufacturers for single therapy. In theory, combination therapy should involve reduction in the doses of all the drugs being used.

2.6.iii Conversion to azathioprine
This approach to the problem of CyA nephrotoxicity has received much attention. Converting drug regimens to conventional therapy with
azathioprine has been investigated in many centres with very variable results. Conversion aims to derive the early benefit of increased graft and patient survival from CyA and then avoid nephrotoxic problems which may result from long term CyA therapy. If nephrotoxicity is amplified by the prolonged effect of CyA on a kidney which has been previously subjected to episodes of dysfunction, withdrawal of this drug will remove the effector from a system which has already been initiated. The greatest problem with this therapeutic manoeuvre is the appreciable incidence of acute rejection which ensues, regardless of the time of conversion. Consequently, no consensus on the optimum time to convert exists.

Several centres have attempted conversion at 3 months post-transplantation. In Leicester (Veitch et al 1987), one third of the patients developed acute cellular rejection or severe infections, whilst another third went on to have late severe rejection episodes. It was felt that the risks of conversion at 3 months outweighed the benefits in this unit. Conversion at 3 months in Oxford still produced a 34% incidence of rejection which responded to steroid therapy or re-conversion, however, the remaining 66% of patients continued to do well (Morris et al 1983). Physiological changes thought to result from CyA also appear to respond to cessation of therapy as demonstrated by Chapman et al (1985). In this study, 50% of patients were hypertensive prior to conversion. Afterwards, blood pressure fell in many of the patients converted to such an extent that the control group, treated with azathioprine and prednisolone from the onset, eventually had an overall higher incidence of hypertension. Falling creatinine levels also demonstrated improvement in renal function.

In Chicago (Thistlewaite et al 1985), 27 renal allograft recipients were
converted for financial reasons 6 months after transplantation from a mean daily CyA dose of 6mg/kg. Only patients with stable function, however, were studied. This was a more suitable time interval for conversion as only 4 (15%) patients suffered an acute rejection episode. This small group, however, did suffer an appreciable loss of renal function as a result, demonstrated by the rise in serum creatinine concentration from a mean pre-conversion level of 1.9mg/dl (168mmol/l) to 2.9mg/dl (256mmol/l) after treatment of rejection. CyA therapy was recommenced in these patients to avoid further episodes of rejection. The remaining 85% underwent successful conversion with improvement of function, mean serum creatinine decreasing to 1.4mg/dl (124mmol/l) on azathioprine. Curtis and co-workers (1986a and b) demonstrated improved renal function in stable patients following conversion 8 months after renal transplantation. A significant increase occurred in ERPF and significant fall in both RVR and MAP yet, no significant change was recorded in serum creatinine concentration; 18% of patients suffered a rejection episode.

Results from conversions performed as late as 1 year post-transplantation still do not indicate the best time to withdraw CyA therapy. The ideal time would be one at which the risk of subsequent graft rejection was minimised and yet prior to the onset CyA-induced deterioration in renal function. In Glasgow, of 45 renal allograft recipients converted at one year, 2 grafts were lost and 3 patients went on to develop an unacceptable severe and progressive deterioration of function (Briggs 1989). Weimar et al (1987) also converting at one year, decided a 12 month time interval was acceptable or even too late as patients with a first renal allograft demonstrated only a 21% incidence of rejection. Nevertheless, it was concluded that caution
should be exercised when dealing with patients who have been sensitised by a previous transplant, as 4 out of 7 patients in this category suffered rejection. All, however, demonstrated an improvement in serum creatinine and creatinine clearance in addition to a fall in BP.

Hunt et al (1987) converted heart transplant recipients as late as 4 years after surgery with 14 out of 15 patients eventually stabilising with improved renal function. 13 of these, however, suffered a rejection episode and even when stabilised on azathioprine, renal function had not returned to normal.

The decision to convert and the optimum time for this proves difficult. If a patient appears well and stable on CyA it is unwise to risk rejection and in some cases graft loss. On the contrary, in favour of conversion, although some histological changes seen are permanent, an element of the functional renal impairment induced by treatment with CyA remains reversible for some time after transplantation. The reversible element is at least amenable to improvement (Myers 1986) but probably does not return function to normal. This has been demonstrated in liver and heart transplant recipients who had healthy kidneys with normal function prior to transplantation, (Hamilton et al 1982, Hunt et al 1987) which did not return to pre-transplant levels. Consequently, conversion to conventional therapy will not completely ameliorate the problem of chronic CyA nephrotoxicity.

2.7 Mechanisms of Cyclosporin A nephrotoxicity

CyA exerts its nephrotoxic effects by causing an acute intra-renal vasoconstriction in response to an oral or intravenous dose of drug. In animal experiments a single dose of CyA causes a rapid and reversible reduction in renal blood flow but as no more than a transient parallel
effect has been noted on systemic mean arterial pressure, intra-renal vascular resistance must become elevated in order to maintain the reduction in perfusion (Paller and Murray 1985). These findings demonstrate the functional effect of an acute dose of CyA, bringing about a rapid physiological response. If CyA were primarily a structural toxin, causing direct damage or death of cells, the response to a single acute dose would tend to be permanent and irreversible rather than transient.

Sullivan et al (1985b) recorded a decrease in renal blood flow in rats within 3 minutes of commencing an infusion of CyA at a dose of 10 mg/kg. The injection of radio-isotopically labelled microspheres has been used experimentally to study blood flow in anaesthetised dogs. In these animals, infused CyA produced a significant fall in renal cortical perfusion when compared with 5% dextrose solution or Cremophor (an oil sometimes used experimentally as a vehicle for CyA). In these unconscious animals it was also noted that most other organs also demonstrated a small reduction in blood flow (McKenzie et al 1985). Most experimental workers have concentrated their investigation on the renal blood flow and systemic blood pressure responses to CyA. Except in the case of the spontaneously hypertensive rat, other conscious animals do not demonstrate sustained increases in MAP in response to CyA therapy. Paller et al (1985) used labelled microspheres in conscious rats and found that at doses of 10mg/kg/d no decrease in RBF occurred, however, at the larger dose of 20mg/kg/d a marked renal vasoconstriction and hypoperfusion occurred. Weir et al (1989) have recently reported the effect of an infusion of CyA in a small group of healthy female volunteers. Despite using a relatively large dose of intravenous CyA, only a non-significant fall in ERPF and rise in RVR was demonstrated.
Hauser et al (1988), however, demonstrated a significant fall in the clearance of PAH in a group of renal transplant recipients taking their first oral dose of CyA. It is possible that the renal allograft is more sensitive to the haemodynamic effects of this drug.

Experimental evidence suggests that a large single dose of CyA given to an animal induces a rapid increase in RVR. Therefore, it is reasonable to hypothesise that repeated doses given in the clinical situation, probably induce a sustained rise in intra-renal vascular resistance which may be directly proportional to the whole blood CyA levels. It is believed that this prolonged vasoconstriction is responsible for the permanent structural damage which can be demonstrated histologically after long term CyA therapy. A chronic increase in RVR has been demonstrated both in animals and in man. Transplant recipients who have their therapy converted from CyA to azathioprine demonstrate a measurable increase in renal blood flow after conversion. This improvement in blood flow and reversal of the nephrotoxic effect of CyA is seen as late as 12 months post-operatively (Hunt et al 1987).

Sullivan et al (1985a) treated rats with CyA for 7 days producing a 15% reduction of RBF, a 13% increase in RVR and a 43% decrease in the clearance of inulin. English et al (1987) performed electron microscope studies of the afferent glomerular arterioles of the rat and was able to demonstrate a progressive narrowing of the afferent arteriole visible from the seventh day of therapy. Photomicrographs demonstrate an obvious narrowing close to the glomerulus whilst measurements made from perfusion casts indicated a 34% reduction in cross sectional area from the fourteenth day of therapy. One problem with the interpretation of this elegant work is the excessively high CyA blood levels, between 3000 and 4000ng/ml, recorded in these animals. Therefore the effects
recorded may not be truly representative of the clinical situation.

Long term studies of patients receiving CyA for renal and other allografts have demonstrated a reduction in GFR and ERPF with a concomitant rise in RVR. In the majority of studies there are, however, no recorded values of pre-operative renal function with which to compare post-transplant values. In the case of the renal allograft recipient, the only available indicator of organ function prior to CyA therapy is the serum biochemistry recorded in the donor prior to nephrectomy.

Wheatley et al (1987) measured GFR and ERPF in 11 patients who had normal renal function, prior to liver transplantation. The same measurements were repeated over a 2- to 26-month period post-operatively whilst on CyA therapy. A 40% fall in ERPF was noted soon after surgery. This value improved with time but despite decreasing CyA doses, later ERPF only maximally attained between 45 and 60% of the pre-operative values. Renal biopsies performed in 6 of these patients showed only moderate vascular changes again demonstrating the functional nature of the "CyA effect". Marked improvement in RBF and renal function has been recorded in such patients when therapy is successfully converted from CyA to azathioprine. However, despite improvement, function does not reach normal values in renal transplant recipients (Curtis et al 1986b) or even in cardiac allograft recipients (Hunt et al 1987) with normal pre-operative renal function. Thus it seems that an irreversible reduction in renal function occurs within the first few weeks after transplantation, which is then compounded by further deterioration which is relatively reversible on cessation of CyA therapy. Several conditions frequently occur in the early post-operative phase of a renal allograft. These include acute cellular rejection, urinary tract infection, ureteric or vascular obstruction. Any of these conditions either alone,
or in combination with the added vasoconstrictor effect of CyA could be responsible for the initial permanent loss of renal function demonstrated. Chronic CyA treatment may then be responsible for the longer term dysfunction which is reversible at least in the early post-operative period.

In conclusion, both animal and human studies provide evidence to support a vasoconstrictive effect of CyA on intra-renal haemodynamics. However, the mechanism by which CyA exerts this effect remains unclear. Several vasoactive systems are involved in renal autoregulation and are able to bring about reductions in renal blood flow and a concomitant reduction in GFR; all are under extensive investigation. To date results suggest that the Renin-Angiotensin system, Adrenergic Nervous system, calcium channels and prostaglandins possibly all play a part. Recently Platelet Activating Factor (PAF) has also been implicated in the aetiology of CyA nephrotoxicity in animals and may also be involved in man.

2.7.i Renin-Angiotensin system

In man renin is released in response to hypovolaemia, detected as a fall in hydrostatic pressure in the afferent arteriole, as a consequence of stimulation of the sympathetic nervous system and finally, in response to a low sodium concentration within the fluid entering the distal tubule. The end product of the Renin-Angiotensin (RA) pathway is aldosterone which enhances sodium resorption in exchange for K⁺ ions in the distal tubule. The whole system is under a continuous negative feedback which maintains equilibrium (Brenner and Rector 1986). Much animal work performed mostly in rats, has been undertaken to investigate the effect of CyA on the Renin-Angiotensin-Aldosterone System (RAAS). The evidence produced indicates stimulation of the RAAS by CyA, in the rat, demonstrated as a rise in plasma renin activity
(PRA). This is measured using a bioassay which relies on the activity of renin in the conversion of angiotensinogen to angiotensin I; the concentration of angiotensin I is then measured by RIA. Consequently, this assay technique measures only active renin, a point that has produced some controversy and will be discussed later.

As early as 1983, work in dogs and rats demonstrated rising PRA in response to CyA therapy (Siegl et al 1983), results which have since been reproduced in many other studies (Perico et al 1986b, Murray et al 1985). The spontaneously hypertensive rat seems particularly sensitive to the effects of CyA and in this animal the increase in PRA is especially marked (Siegl et al 1983). It is possible that the exaggerated release of renin and hence angiotensin II, a powerful vasoconstrictor, is responsible for the rising BP seen in these animals but not demonstrated in other species of rat. This hypothesis is supported by the work of Lustig et al (1987) who found that captopril, an inhibitor of angiotensin I converting enzyme, significantly reduced the hypertension produced by CyA in this particular species of rat. Unfortunately, no effects on renal function were recorded. In other studies, however, captopril did not seem to be effective in reversing or improving the renal effects of CyA. Murray and co-workers (1985) found this drug had no effect on the CyA-induced fall in RBF or rise in RVR demonstrated in non-hypertensive Sprague Dawley rats and no effect was noted by Dieperink et al (1986a) also in CyA toxic rats. Perhaps there is an intrinsic problem with the drug captopril rather than its mode of action, as enalapril, a related compound gave quite different results. Thomson et al (1987) noted that enalapril treatment caused an increase in GFR and urinary excretion of sodium in addition to a fall in N-acetyl D-glucosaminidase (NAG) enzymuria, a feature thought to indicate a toxic
effect of CyA on the proximal renal tubule.

In view of the difficulty encountered in attempting to block the renin response and ensuing haemodynamic changes, it is more likely that elevation of PRA may be a secondary phenomenon and not a direct effect of CyA. Ryffel and Mihatsch (1986) and many others continue to record the anorexia and weight loss associated with the toxic doses of CyA given to experimental animals and suggest that renin is released in response to both salt and water deficiency. However, in a study in which CyA toxic rats received diets containing variable amounts of sodium, no significant difference in plasma renin content was recorded in any group (Gerkens et al 1984). However, despite this it was interesting that those receiving a low salt diet demonstrated exacerbated nephrotoxicity with significantly higher serum creatinines and significantly lower creatinine clearances, when compared with controls. This occurred despite no higher circulating levels of PRA, indicating the possibility of another intermediary system which is sensitive to plasma sodium concentrations. Perico et al (1986b) specifically noted that serum sodium levels in animals given CyA for 45 days were comparable with controls, which would tend to discount an hyponatraemic stimulus for the elevated plasma renin found in these animals.

The increases in plasma renin recorded after CyA therapy in rats are associated with comparable significant elevations of urinary thromboxane B₂, both of which precede the fall in GFR and rise in serum creatinine (Perico et al 1986b). Both angiotensin II and TXA₂ can contract mesangial cells in addition to vascular smooth muscle. This combined effect would reduce glomerular surface area and contribute a further possible mechanism to the reduction of ultra-filtration capacity seen
during CyA therapy.

The RA system is intimately involved with prostanoid production both as a stimulator and an effector. Cultured glomerular epithelial cells, stimulated with arachidonic acid, produce a variety of prostaglandins whilst these in their turn can bring about renin release from the juxtaglomerular apparatus. Secondly, treating these cultured cells with angiotensin II leads to prostaglandin production (Beierwaltes et al 1980). CyA is known to produce an imbalance in vasoconstrictory and vasodilatatory renal prostaglandin production with emphasis on the former and it may be that this effect is closely inter-linked with angiotensin production in the animal models described.

Despite the strong evidence for CyA stimulation of PRA in animals, no comparable evidence exists for the same mechanism in man. In heart transplant recipients with native kidneys, 1 to 2 years after transplantation when GFR was reduced by 50%, plasma renin activity was found to be lower than in normal people (Myers et al 1984). Jorkasky et al (1987) measured PRA in renal transplant recipients receiving either CyA or azathioprine and found significantly lower levels in the CyA treated patients. People have even attempted to stimulate the RA system in CyA treated patients, by potassium loading (Nath et al 1984) or low sodium diets (Bantle et al 1985) and yet PRA still remains low. As previously mentioned, it has been suggested (Mason 1989) that the full picture is not emerging as total plasma renin concentration may not be measured by bioassay. However, recently Stanek et al (1987) used a RIA for direct total renin measurement in 11 renal transplant recipients over a 12 month period after transplantation. Plasma renin was measured 2 hours after a dose of CyA and no elevation of renin, aldosterone nor arginine vasopressin was noted at any time, neither did captopril
significantly alter these findings.

The conclusion must be that in the human situation the RAAS is not activated by CyA although in the animal model it appears to play an important role in CyA nephrotoxicity.

2.7.ii Calcium Channels

Calcium is intimately involved in cell metabolism and respiration, in addition to being responsible for contraction of all types of muscle. Mesangial cells from the kidney glomerulus contract in response to angiotensin II in culture but only in the presence of calcium ions, an effect which can be inhibited by calcium-channel blockers (Sraer et al 1987).

Several conditions lead to an intra-cellular influx of calcium, most notably tissue ischaemia, during reflow perfusion following a period of ischaemia (Wagner et al 1987) and also in response to CyA therapy (Feehally and McNally 1989). Therefore, in the renal transplant situation a combination of these factors exists tending to raise cytosolic Ca^{++} levels, leading to mitochondrial dysfunction, impaired cell respiration and even cell death (Wagner et al 1987). CyA itself binds intra-cellular calcium-binding proteins, leading indirectly to vascular smooth muscle contraction (English et al 1987). All these deleterious effects can to some extent be reversed by calcium-channel blocking drugs.

In the absence of CyA, nifedipine appears to confer a protective benefit on renal function. Treating mild hypertension with nifedipine led to a significant increase in GFR and ERPF with a concomitant fall in RVR even in patients in whom BP did not respond to the therapy (Reams et al 1988). The inhibition of rising cytosolic ionised Ca^{++} concentration
was thought to reduce vascular sensitivity to other vasoreactive agents such as angiotensin II and nor-adrenalin.

In experimental models of CyA toxicity, the protective effect of calcium-channel blockade has been demonstrated. In an isolated perfused kidney model, verapamil infused prior to CyA led to maintenance of pre-therapy GFR, perfusate flow rate and urine output. Normally after CyA infusion this model develops toxic changes within one hour of treatment (Sumpio et al 1987). Dogs, baboons and rats treated with CyA and a calcium-channel blocker show an improvement in creatinine clearance when compared with controls, (Bunke and Wilder 1988), whilst the onset of CyA nephrotoxicity is delayed but not prevented by treatment with verapamil (Sumpio et al 1987). Dieperink and co-workers (1986a) demonstrated a rise in GFR and significant improvement in renal function in CyA-toxic rats given nifedipine when compared with those given prostacyclin, indomethacin or phenoxybenzamine.

Calcium-channel blockers have also been demonstrated to protect against the effects of CyA in transplant recipients. A retrospective study demonstrated lower serum creatinine levels in patients treated with nifedipine for their hypertension following renal transplantation than in patients given other antihypertensive agents (Feehally et al 1987). A prospective study giving one month of nifedipine treatment to normotensive allograft recipients is presently underway in Leicester.

Patients receiving CyA have an enhanced thromboembolic tendency. It has been noted that platelets from patients treated with CyA demonstrate increased aggregability ex vivo, however, in patients also taking nifedipine this tendency to aggregate was absent at trough CyA levels and blunted at peak levels (Grace et al 1987).
Pre-treatment of the donor kidney with a diltiazem infusion prior to transplantation plus treatment of the recipient at the time of transplantation and for a short period post-operatively significantly decreased the incidence of post-transplantation acute tubular necrosis. This also produced a slight but non-significant increase in GFR, RBF and decrease in serum creatinine when compared with controls. However, the benefit of this short term calcium-channel blockade was not maintained, at 6 months no difference existed between the two groups (Wagner and Neumayer 1987). Therefore, early short term calcium-channel blockade appears to protect the kidney from early ischaemic insult but long term, other mechanisms come into force.

Calcium ion flux is also intimately involved with prostaglandin synthesis. Thromboxane A₂, which is produced in large quantities by the kidneys of both CyA treated animals and patients, seems to act by directly releasing calcium ions from intra-cellular sources producing an adverse rise in cytosolic calcium concentration. This effect is also produced by U44069, a thromboxane-mimetic derived from the endoperoxide precursors of TXA₂ (Jaschonek and Muller 1988). The effects of U44069 can also be blocked by calcium-channel blockade experimentally (Loutzenhiser and Epstein 1987).

Prostacyclin is known to work as a vasodilator and platelet inhibitor by increasing intra-cellular concentrations of cAMP. This latter compound is intimately involved in preventing the influx of ionised Ca++. There is evidence to suggest that prostacyclin formation may be reduced in CyA nephrotoxicity and as a result the induction of cAMP is inhibited and extra-cellular calcium ions enter the cells unimpeded. However, treatment with a calcium-channel blocker can prevent this entry of ionised Ca++ by a mechanism which does not involve cAMP (Fantone and
In conclusion, the movement of calcium ions seems to play an important role in the aetiology of CyA nephrotoxicity. Evidence does suggest, however, that an imbalance of prostanoid production may be the primary event which then exerts its effects via the movement of ionised calcium across cellular membranes.

2.7.iii Sympathetic nervous system

The majority of work investigating the possibility of sympathetically mediated CyA-induced nephrotoxicity is derived from animal studies. In a complex animal model, impulses were recorded from the cut ends of efferent and afferent rat renal nerves in situ. Both efferent and afferent activity was increased by up to 80% in response to a dose of CyA although no change in renal blood flow was noted. However, a prompt but transient rise in heart rate and systemic BP occurred, lasting only 20 minutes, whilst in the contralateral, normally innervated kidney a decrease in GFR but not RBF was demonstrated (Moss et al 1985). Problems arise with a preparation of this nature, in determining which effects are purely secondary to drug and which result from the obviously stressful nature of the experimental model. However, the relatively larger decrease in GFR compared with RBF in the normally innervated kidney reflects results found by other worker in studies with intact animals (Perico et al 1986a). Physiologically, the denervated kidney perhaps more closely resembles a renal allograft yet does not appear to be as sensitive to the direct effect of CyA. As measured nerve activity was significantly increased, the overall conclusion was that CyA is "sympathoexcitatory". In support of this finding, Garr and Paller (1988) demonstrated an enhanced vasoactive response to exogenous nor adrenalin in the denervated kidneys of rats treated with CyA, whilst

A sympathoexcitatory effect of CyA might explain the improvement in renal function produced by blockade of the sympathetic nervous system of intact rats rendered CyA-toxic. Renal denervation or alpha adrenergic blockade with phenoxybenzamine inhibited the decrease in ERPF, GFR and rise in RVR normally seen in rats given acute doses of CyA (Garr and Paller 1988, Paller and Murray 1985). However, Dieperink and co-workers (1986a), performed a similar experiment and found that even with small doses, phenoxybenzamine dropped systemic BP below the autoregulatory level for the kidney and therefore no increase in renal blood flow occurred. Therefore available evidence is far from conclusive.

Vincent et al (1987a) treated kidney transplant recipients with Hydergine (an alpha\textsubscript{1} and alpha\textsubscript{2} adrenoceptor antagonist) and noted a rapid fall in serum creatinine. As a detectable decrease in systemic BP took between 4 and 8 weeks to develop, the improvement recorded in renal function clearly was not occurring in response to systemic haemodynamic effects. Curtis et al (1986a) also noted that in renal transplant patients the rise in RVR is not seen until 2 weeks after transplantation. They postulated that the sympathetic nervous system could not be directly responsible for decreased RBF and GFR as re-innervation would not have occurred by this time. A possible synergistic effect between sympathetic amines and other mediator substances might provide a better explanation. The evidence from clinical work suggests it is unlikely that the sympathetic nervous system is a direct mediator of the toxic effects of CyA in the human denervated renal allograft. The possibility of its involvement in enhancing the renal response to other vasoconstrictor influences cannot,
however, be excluded.

2.7.iv Prostaglandins
Renal tissue contains all the enzyme systems necessary for the synthesis of prostaglandins. These substances are autacoids, produced local to their site of action. Measuring levels in blood or plasma gives a poor indication of local tissue concentrations within any organ. Urinary levels of prostanoid metabolites are used to provide a better but not necessarily reliable indicator of renal prostaglandin production.

CyA tends to enhance the production of the vasoconstrictor prostaglandin thromboxane A$_2$ which is detected as its stable urinary metabolite thromboxane B$_2$ (TXB$_2$). Evidence also suggests that in response to CyA the production of vasodilatory prostanoids in particular, prostacyclin (PGI$_2$) is reduced. TXA$_2$ is synthesised in large quantities by activated platelets and is also involved in their further stimulation leading ultimately to aggregation and adhesion to vessel walls. Normally a fine balance exists between vasodilator and vasoconstrictor prostanoids and hence also platelet activity is carefully controlled. In the presence of CyA, this balance is weighted strongly in favour of increased platelet adhesion and aggregation in addition to vascular smooth muscle contraction. The overall effect could well provide an explanation for the mechanism of the toxic effects of CyA. Prostaglandins and the effects of CyA on their production will be discussed in detail in sections 2.8 and 2.9.

2.7.v Tubular toxicity
Evidence suggests that CyA affects renal blood flow and filtration capacity. This may directly or indirectly lead to an alteration in the
delivery and handling of water and electrolytes within the proximal and distal tubules, affecting clearance values for many substances. Clinically this is seen as sodium and water retention and occasionally an hyperkalaemic acidosis. Some authors feel that these changes occur secondarily to altered renal haemodynamics both within and outside of the glomerulus (Dieperink et al 1983, Vincent et al 1987b). Others believe that CyA has a direct toxic effect upon the tubules in addition (Whiting et al 1982).

Gamma glutamyl transferase (GT) and more commonly N-acetyl-beta-D-glucosaminidase (NAG) are enzymes released by the brush border and lysosomes respectively of damaged renal tubular cells and are easily detected in the urine. In addition, fine needle renal biopsy can be used to demonstrate gross structural damage to tubular cells. Some workers have demonstrated the uptake of CyA into tubular cells and then postulated this as part of the toxic mechanism (Nagineni et al 1987). Yet Ryffel and Mihatsch (1986) examined both rat and human renal tissue and were unable to detect CyA in either at therapeutic doses.

In 1980(b), Whiting et al treated Sprague Dawley rats with relatively small daily doses of between 12.5 and 25 mg/kg of CyA, in an attempt to produce either toxic or immunosuppressive effects. Histology, however, remained normal, urinary NAG levels were not elevated whilst immunosuppression was incomplete as shown by a reduced immune response to sheep erythrocytes. In 1982, the same group produced NAG enzymuria and light microscopic changes by giving larger doses of CyA (25 and 100 mg/kg/d) for more than 7 days. The toxicity of larger doses of CyA has also been demonstrated by other workers (Thomson et al 1987, Pomer et al 1987).
NAG enzymuria is an indicator of renal tubular damage but is not specific for CyA. Frusemide alone can produce enzymuria, whilst in transplant recipients it was more commonly seen in patients taking azathioprine (Sullivan et al 1985a). In the rat model, large doses of CyA are thought to induce persistent NAG enzymuria. Contrary to this belief, Whiting and co-workers (1983) discovered NAG levels improving to normal after 4 to 6 weeks of continuous treatment with 25mg/kg/d. This was accompanied by improvement of serum creatinine and was thought to result from hepatic enzyme induction increasing CyA metabolism. However, this does not explain why NAG levels increased after this period of recovery despite no alteration in the dosage regimen and again casts doubt upon CyA acting as a direct tubular toxin. English et al (1987) treated rats with CyA until blood concentrations ranged between 3000 and 4000ng/ml; despite showing both histological and functional toxic changes, these animals demonstrated no NAG enzymuria. High concentrations of CyA in cell culture are known to be cytotoxic (Zoja et al 1986), while at these concentrations, the production of NAG may be inhibited or exhausted. A direct relationship between NAG enzymuria and experimental CyA therapy in animals cannot be assumed.

In man there is also little evidence for NAG enzymuria being a selective indicator of CyA therapy or toxicity. Nephrotoxins, ischaemia and renal allograft rejection can all lead to elevation of urinary NAG levels, with those demonstrated during rejection tending to return to normal on steroid therapy (Whiting et al 1980a). Finn and Gitelman (1984) studied 10 patients who received between 6 and 14mg/kg/d of CyA in the post-transplant period. They found that although NAG levels were elevated in the early post-operative days, these decreased as function improved and stayed within the normal range once function was stable, despite CyA
blood levels as high as 1000ng/ml. Similar patterns of urinary NAG concentration were also demonstrated in a group of patients treated with azathioprine, suggesting that NAG enzymuria in the renal transplant recipient may result from a variety of factors which affect renal and also tubular function, rather than a direct specific effect of CyA therapy.

In animals, micropuncture studies of single nephron function have been performed to investigate the tubular handling of water and electrolytes. Measurement of renal solute clearances has been undertaken before and after starting CyA therapy. The clearance of lithium is a useful value which closely equates with the delivery of tubular fluid from the end of the proximal straight segment. The handling of lithium also parallels that of sodium. In rats given 50mg/kg/d of CyA, a significant decrease in the clearance of lithium, sodium and potassium was associated with NAG enzymuria, glycosuria and increased urine flow rate. This was interpreted as demonstrating the tubulotoxic effect of CyA in combination with a haemodynamic effect on the afferent arteriole, possibly mediated via the RA or prostaglandin system (Whiting and Simpson 1988). This view is supported by Tonnesen et al (1983) who found that an acute dose of CyA decreased the absolute and fractional excretion of both Na\(^+\) and K\(^+\) ions. In this study the effect of CyA was improved but not corrected by plasma expansion. However, Dieperink et al (1986b) gave similar doses to rats and suggested a different explanation for their findings. In this study, proximal fractional resorption of ions was actually increased, whilst the morphology of both proximal and distal tubules remained normal with no evidence of vacuolation or damage. In these animals, CyA therapy caused neither tubular damage nor impaired tubular function. It was also found that a
moderate intravenous saline load immediately corrected the depression in GFR indicating that the effect of CyA was acute and reversible and more likely to result from a functional effect on glomerular haemodynamics. In addition, the absence of rising intra-tubular pressure which would accompany impaired water resorption led to the final conclusion that CyA was not a tubular toxin and that relative tubular function had actually improved on CyA therapy. Humes et al (1985) gave rats 100mg/kg/day of CyA and produced a sharp decline in renal function; again no evidence for CyA induced toxic renal tubule cell injury could be demonstrated. More recent work by Mason (1989) has demonstrated that despite a dose-dependent decrease in GFR by day 5 of CyA therapy there was only an insignificant minimal loss of sodium and water resorption.

Measured alterations in the tubular handling of sodium and water in the rat occur in the presence of elevated circulating levels of renin. In these animals, both enalapril and prostaglandin E₂ (PGE₂) improved GFR and also reduced urinary NAG concentrations (Thomson et al 1987, Pomer et al 1987). Enalapril is an angiotensin I converting enzyme inhibitor whilst PGE₂ is a vasodilator. This would suggest that CyA exerts its toxic effect on the renal tubules via a vasoconstrictory mechanism related to the elevated renin and TXA₂ levels, rather than by a direct effect on tubular cells. In addition, a feedback mechanism originating in the renal tubules cannot be excluded.

Glomerular function can also be modified by variations in the concentrations of filtered substances delivered to the distal tubule; this mechanism is known as "Tubulo-glomerular feedback". Therefore altered tubular handling of electrolytes and water can also affect intra-renal blood flow. For example, decreased proximal resorption of a solute leads to increased distal delivery of fluid and solute which in
turn produces reflex vasoconstriction and a decrease in GFR (Schnerman et al 1984). Gerkens et al (1984) found that a low salt diet in rats exacerbated CyA nephrotoxicity and suggested that increased delivery of chloride ions to the distal tubule during salt deprivation might be responsible for this.

In man, no comparable increase in renin secretion has been demonstrated during therapy with CyA; it is therefore unwise to extrapolate directly from animal studies. While tubular studies of man have demonstrated variable changes in electrolyte handling, micropuncture studies have not been performed. In the early post-transplant period, Morales et al (1987) demonstrated decreased fractional excretion of sodium and high urinary creatinine concentrations in patients taking CyA when compared with a similar group taking azathioprine. When tubular function is impaired by acute tubular necrosis, urinary sodium concentration is elevated, thus these findings do not correlate with the effects of a tubular toxin (Morales et al 1988). This hypothesis is supported by the work of Grace (1988) who concluded that the increased fractional resorption demonstrated by patients taking CyA was highly suggestive of a tubule which was functionally intact but which was being underperfused.

2.8 Prostaglandins
It has already been stated that renal tissue, in particular the medulla, is rich in the enzyme systems required to synthesise prostaglandins. These substances are produced close to their site of action and hence the measurement of plasma concentrations is an unreliable indicator of tissue levels (Dunn and Hood 1987). Urinary concentrations may be more indicative of renal prostanoid production, however, seminal contamination in men can provide an additional source of error (Patrono
and Dunn 1987).

Prostanoids are synthesised from the unsaturated 20 carbon fatty acid, arachidonic acid, which is released from cell membrane phospholipids mainly by the action of the enzyme phospholipase A\(_2\) (PLA\(_2\)). This enzyme is inhibited by corticosteroids which act via the production of an inhibitory protein, lipocortin (Patrono and Dunn 1987). Cyclo-oxygenase (COO) is the rate-limiting enzyme system which converts arachidonic acid to the endoperoxides PGG\(_2\) and PGH\(_2\) (prostaglandins G\(_2\) and H\(_2\)), the precursors of all prostanoids. Endoperoxides are unstable, having very short half-lives but are active substances in their own right, PGH\(_2\) being a powerful thromboxane receptor agonist (Halushka et al 1987). The endoperoxides are acted upon by specific synthases to form individual prostanoids. (Fig 2.1)

The most important prostanoids linked with CyA activity are thromboxane A\(_2\) (TXA\(_2\)) and prostacyclin (PGI\(_2\)). TXA\(_2\) was first identified by Hamburg et al in 1975. It is synthesised largely by platelets and also within certain tissues including kidney and lung (Bunting et al 1983). It is a powerful constrictor of vascular and smooth muscle and also stimulates platelet aggregation and adhesion. Once platelets are activated, they synthesise and release further quantities of TXA\(_2\) in a self-potentiating amplification cascade.

PGI\(_2\) was first described by Moncada et al in 1976. This prostaglandin is synthesised by vascular endothelial cells and is both a vasodilator and a potent inhibitor of platelet activity (Bunting et al 1983), suggesting an action which is pharmacologically opposite to that of TXA\(_2\) (Whittle and Moncada 1983). Both substances have a short half life, 3 min for PGI\(_2\) and 30sec for TXA\(_2\), and cannot be measured in their native
Membrane phospholipids
  \[ \rightarrow \text{phospholipase } A_2 \]
  \[ \rightarrow \text{arachidonic acid} \]
  \[ \rightarrow \text{cyclo-oxygenase} \]
  \[ \rightarrow \text{PGG}_2 \]
  \[ \rightarrow \text{peroxidase} \]
  \[ \rightarrow \text{PGH}_2 \]

- **thromboxane synthase**
- **endoperoxide isomerase**
- **prostacyclin synthase**

- **THROMBOXANE (TXA\(_2\))**
- **PROSTACYCLIN (PGI\(_2\))**

\[ \rightarrow \text{TRA} \]
\[ \rightarrow \text{thromboxane } A_2 (\text{TXA}_2) \]
\[ \rightarrow \text{PGD}_2 \]
\[ \rightarrow \text{PGE}_2 \]
\[ \rightarrow \text{PGF}_{2\alpha} \]
\[ \rightarrow \text{hydrolysis} \]
\[ \rightarrow \text{TXB}_2 \]
\[ \rightarrow \text{hydrolysis} \]
\[ \rightarrow 6k\text{PGF}_{1\alpha} \]

**FIG. 2.i** The Prostaglandin Synthetic Pathway - showing potential sites of manipulation.

**NSAID** = Non-steroidal anti-inflammatory drug

**TSI** = Thromboxane synthase inhibitor

**TRA** = Thromboxane receptor antagonist
forms. Both are hydrolysed to the more stable metabolites, 6-keto prostaglandin F\textsubscript{1} alpha (6-keto PGF\textsubscript{1a}) and TXB\textsubscript{2} respectively, which can be measured by radioimmunoassay.

Cell and tissue culture methods have been widely employed to determine the relative production of prostanoids by different types of renal tissue. Most evidence arises from studies of rat tissue. It is difficult to avoid "artefactual" stimulation of prostaglandin production and this must be borne in mind when interpreting results from any physiological model. In tissue culture PGE\textsubscript{2} is the principal product of stimulated rat glomeruli (Sraer et al 1982) with lesser quantities of PGI\textsubscript{2} and TXA\textsubscript{2} also being released (Scharschmidt et al 1986, Petrulis et al 1981). Glomeruli produce at least ten times the quantity of prostanoids when compared with mesangial cells in culture (Kreisberg et al 1982). The major prostanoid produced by human glomeruli in culture is PGI\textsubscript{2} (Scharschmidt et al 1983), again in addition to small quantities of TXA\textsubscript{2} when stimulated by arachidonic acid. It is known that PGI\textsubscript{2} is produced almost entirely by vascular endothelial cells (Brenner and Rector 1986). Cultured bovine aortic endothelial cells produce large quantities of PGI\textsubscript{2} either spontaneously or after mechanical or chemical stimulation (Gryglewski et al 1986, Ingerman-Wojenski et al 1981). Depending upon the species and site of origin of vascular endothelial cells, again, different prostanoids are produced in different relative quantities.

When stimulated, rat and human vascular endothelial cells isolated from fat microvessels produce PGE\textsubscript{2}, human saphenous vein PGF\textsubscript{2a} whilst human umbilical vein endothelial cells (HUVEC's) produce large quantities of PGI\textsubscript{2}, detected as the metabolite, 6kPGF\textsubscript{1a}, in the supernatant (Taylor et al 1987). It has been noted that foetal endothelium tends to produce
more PGI$_2$ than adult vessels (Remuzzi et al 1979). These cells are readily available and relatively easy to harvest and grow in culture and being a rich source of PGI$_2$, provide a very useful human experimental model (Bjoro et al 1986).

Platelets are the major source of TXA$_2$ production, however, evidence suggests that it is also synthesised in small quantities by other tissues in the absence of platelets. For example, arachidonic acid stimulation of cultured bovine aortic endothelial cells (Ingerman-Wojenski et al 1981) yielded detectable concentrations of 6kPGF$_{1a}$ and TXB$_2$ in a ratio of 10:1. Human umbilical artery, free of all platelets on electron microscopic inspection, also released TXB$_2$ when stimulated. As this can be inhibited by indomethacin, TXB$_2$ must be synthesised locally prior to release. Bjoro and co-workers (1986, et al 1986) describe this phenomenon believing the production of PGI$_2$ and TXA$_2$ in this preparation to result from "mutual stimulation". This suggestion may have a bearing on the physiological relationship which exists between these two substances in vivo. Platelet-rich plasma (PRP), provides a widely used ex vivo experimental model for TXA$_2$ production.

Scharschmidt et al (1986) demonstrated that certain renal prostanoids induce contraction of both smooth muscle and mesangial cells in tissue culture. TXA$_2$ and endoperoxides in glomerular culture produced a decrease in glomerular planar surface area, an effect which is opposed by a thromboxane receptor antagonist or PGE$_2$. Reduction of glomerular surface area may contribute to the reduction in ultra-filtration coefficient produced by vasoconstrictor influences in response to CyA therapy.

In healthy people, prostanoids are produced only in very small
quantities by the kidney and appear to have no important effect on renal function. This is demonstrated by the administration of non-steroidal anti-inflammatory agents. These drugs are potent inhibitors of the COX enzyme system, blocking prostanoid synthesis and yet producing no change in renal function in healthy people (Brenner and Rector 1986).

Clinical conditions associated with systemic vasospasm or thrombosis are characterised by elevated circulating levels of TXB$_2$. Patients with severe arterial disease, Raynaud’s phenomenon and systemic sclerosis not only have high plasma TXB$_2$ levels but also demonstrate elevated levels of 6kPGF$_{1a}$. This suggests a physiological response to elevated TXA$_2$ levels, however, the overriding effect is one of vasoconstriction (Wilkinson et al 1989, Reilly et al 1986).

The prostanoid "balance" is altered in many other conditions and also in animal models of renal disease. For example, in the murine model of rat serum nephrotoxic nephritis, there is increased production of TXB$_2$ and also PGE$_2$, a vasodilator (Scharschmidt et al 1983, Lianos et al 1983). Elevated urinary TXB$_2$ levels are found in association with ureteric obstruction (Yarger et al 1980) and systemic lupus erythematosus in women (Patrono et al 1985). In the renal transplant recipient TXB$_2$ can be elevated by CyA therapy (Jorkasky et al 1987), acute allograft rejection and also in association with deep venous thrombosis and pulmonary embolism (Foegh et al 1981, Ogletree 1987). The elevated concentrations of vasodilator prostanoids detected in these conditions may provide a balance which is protective of renal function (Patrono et al 1985, Verstraete 1985). When these patients receive NSAID’s, the synthesis of all prostanoids is inhibited. Deterioration in both RBF and GFR suggests that renal function exists under prostanoid-induced "vasodilatatory drive" in these people. This effect of NSAID’s has been

PGE$_2$ and PGI$_2$ production is stimulated in the presence of other vasoconstricting influences, for example, angiotensin II (Beierwaltes et al 1980), nor adrenalin (Felgen et al 1977), bradykinin and AVP (Dunn and Hood 1977) in addition to providing a vasodilatory counterbalance to constricting prostanoids. Therefore, in patients with impaired renal function secondary to certain disease processes, maintenance of GFR and RBF relies upon a COO-dependent production of vasodilatory prostanoids (Cibattoni et al 1984). Whilst in conditions characterised by the production of vasoconstrictor substances the vasodilatory prostanoids again act to attenuate the effects of these influences within the kidney (Anderson et al 1976).

A prostaglandin balance, occurring between TXA$_2$ and PGI$_2$ in man, appears to be responsible for the maintenance of intra-renal vascular tone. An imbalance in the intra-renal synthesis of these substances has been proposed in patients taking CyA, producing the characteristic features associated with nephrotoxicity. CyA appears to upset this balance in a way which leads to enhancement of prostanoid-controlled vasoconstrictor activity.

2.9 The effect of cyclosporin A on prostaglandin production
The effect of CyA therapy on prostanoid production has been investigated in a wide variety of *in vitro* and *in vivo* experimental models. By detecting changes in prostanoid concentration a mechanism for CyA nephrotoxicity has been proposed. Consequently, methods to counteract and treat the renal dysfunction produced are undergoing further
As CyA therapy induces a thromboembolic tendency in clinical practice, it was felt that endothelial integrity, PGI2 production and normal platelet function might all be impaired by this drug. Experimental evidence from endothelial cell culture demonstrates that CyA has marked effects on prostanoid production. Zoja et al (1986) treated bovine aortic endothelial cells with very large, non-therapeutic, doses of CyA for long periods. This produced cytolysis, cell disruption and cell detachment in association with rising levels of both TXB2 and 6kPGF1alpha in the supernatant. No other workers have demonstrated CyA-induced cellular damage visible at the light microscope level. However, Brown et al (1986) detected elevated circulating concentrations of Factor VIII-related antigen in a group of nephrotoxic renal transplant recipients taking CyA. This substance is a specific cell marker produced by vascular endothelium and is released in conditions characterised by cell damage. In rabbit aortic strips, Neild et al (1983) also noted that CyA reduced the production of a prostacyclin stimulating factor, thought to be essential for the synthesis and release of PGI2. These results suggest that therapeutic concentrations of CyA, may affect endothelial cell function with only minimal structural damage. Human umbilical vein endothelial cells cultured in the presence of CyA demonstrated decreased synthesis of both basal and calcium ionophore stimulated 6kPGF1alpha (Voss et al 1988). Brown and Neild (1987) showed this CyA-induced inhibition of PGI2 synthesis to be both time and dose-related, whilst Gordon et al (1987) showed it to be associated with a reduction of intra-cellular protein synthesis. In the presence of reduced or absent PGI2 production platelet adhesion and aggregation is enhanced leading to localised thrombus formation. This
effect has been demonstrated in HUVEC culture (Trifillis and Hall-Craggs 1986).

As platelets are the major source of TXA₂, platelet-rich plasma has been used to investigate prostanoid production in patients receiving CyA. An increased tendency to aggregate was demonstrated on stimulation ex vivo. The same platelets also released significantly larger quantities of TXB₂ during activation (Grace et al 1988) when compared with controls. Jorkasky et al (1989) incubated human platelets with CyA in vitro and found that a similar effect was produced only after a 3 hour incubation period.

Despite demonstrating prostanoid production by renal cell cultures, there is little evidence for the effects of CyA upon these preparations. Benigni et al (1988b), however, demonstrated that isolated rat glomeruli converted arachidonic acid to TXB₂ when challenged with CyA. The same investigators also showed that the isolated perfused rat kidney (IPRK) produced more TXB₂ relative to 2,3-dinor TXB₂ in response to CyA suggesting that total TXB₂ measured in urine arises both from platelets and renal tissue (Benigni et al 1988a). The majority of PGI₂ arises from endothelium, but one cannot discount a renal cell contribution to the concentrations measured.

In the IPRK model, most workers have demonstrated an increase in TXA₂ production in response to treatment with CyA (Benigni et al 1988b, Sinzinger et al 1987). In the latter study, in contrast to cell culture models, the production of PGI₂ was also increased.

Increased urinary excretion of TXB₂ is well documented in rats treated with CyA (Coffman et al 1987, Perico et al 1986a and b). The site of production of this metabolite, however, remains unclear. Circulating
levels of TXB$_2$ have not been elevated in studies to date, suggesting localised intra-renal synthesis of this substance.

More recently, however, with more specific assays, Benigni and co-workers have detected significantly increased concentrations of both TXB$_2$ and 2,3-dinor TXB$_2$ in the urine of CyA treated animals (1988a and b). As 2,3-dinor TXB$_2$ is thought to be a metabolite of platelet TXA$_2$ production, these results suggest both a renal and platelet source for the elevated TXA$_2$ synthesis in the rat but with platelets being specifically activated while circulating through intra-renal vessels.

A reduction in PGI$_2$ production in rats treated with CyA has not been demonstrated. Coffman et al (1987) found increased synthesis of PGE$_2$ and PGI$_2$ in rats treated with 50mg/kg/d of CyA, a fact also noted by Murray and co-workers (1985). In contrast, Perico et al (1986a and b) demonstrated no elevation in synthesis of vasodilatatory prostanoids in rats given CyA.

The majority of work performed in man supports increased renal excretion of TXB$_2$ in patients receiving CyA (Jorkasky et al 1987, Kho et al 1988, Weir et al 1989). However, the effect of CyA on PGI$_2$ synthesis in man is less clear. Deray et al (1988) demonstrated a significant decrease in the urinary excretion of both TXB$_2$ and 6kPGF$_{1a}$ in non-transplant patients receiving CyA for autoimmune uveitis. Other workers have found decreased (Kho et al 1988), normal (Jorkasky et al 1987) and elevated (Weir et al 1989) urinary concentrations of 6kPGF$_{1a}$ in separate studies which otherwise all demonstrated increased TXB$_2$ production.

In an intact animal or man, prostanoid production is under many physiological constraints. It is possible that cell culture demonstrates the effect of CyA on a single cell type, whilst in vivo, a
rise in vasodilatatory prostanoids occurs as a protective secondary phenomenon in response to increased TXB\textsubscript{2} or other vasoconstricting stimulus within the kidney. Alternatively, urinary 6kPGF\textsubscript{1a} levels may not reflect true renal tissue production and concentration of PGI\textsubscript{2}.

Finally it is known that vasodilatatory prostaglandins promote the renal excretion of sodium and water (Patrono and Dunn 1987). Patients taking CyA have a greater tendency to develop salt and water retention, a feature which may be indirectly related to reduced intra-renal production of prostacyclin.

It is not known at which site CyA acts in the prostaglandin synthetic pathway. Using HUVEC's, Voss et al (1988) demonstrated that CyA had a non-lethal mode of action as its inhibitory effect on PGI\textsubscript{2} production was lost within 24 hours of a single dose. Phospholipase A\textsubscript{2} is responsible for the release of arachidonic acid from membrane phospholipids; the addition of arachidonic acid to a CyA toxic system of HUVEC's does not enhance PGI\textsubscript{2} production, suggesting that the pathway is blocked distal to this step. As CyA increases the production of one prostanoid, TXA\textsubscript{2} and also reduces the production of another, PGI\textsubscript{2}, it may possibly act at more than one site in the prostaglandin synthetic pathway.

If TXA\textsubscript{2} production is the primary event which occurs in response to CyA therapy, production of PGI\textsubscript{2} may be a secondary and less powerful reactive phenomenon. This relationship is not clearly demonstrated by measuring concentrations of its metabolite, 6kPGF\textsubscript{1a}, excreted in urine. Increased intra-renal synthesis of the powerful vasoconstrictor TXA\textsubscript{2} is therefore considered to be an important factor contributing to the nephrotoxicity of CyA.
2.10 Theoretical approaches to modifying the CyA-induced prostaglandin imbalance

The available evidence suggests that CyA exerts its nephrotoxic effect by inducing an imbalance of intra-renal prostanoid production. It should be possible, therefore, to correct this imbalance using drugs which are known to have activity at specific sites within the prostaglandin synthetic pathway.

The overriding vasoconstrictory effect of excess TXA$_2$ synthesis could be theoretically abolished by blocking either the production or the effects of this substance. Thromboxane synthase inhibitors affect both platelet and renal synthesis, whilst specific receptor antagonists block the effects of TXA$_2$ at its site of action. Alternatively, to counteract the increased vasoconstrictory activity stimulated by CyA therapy, the circulating and intra-renal concentration of vasodilatatory prostanoids could be enhanced by stimulating PGI$_2$ production or by treatment with exogenous prostanoids of similar activity. A combination of these mechanisms is a further possibility.

2.10.1 Cyclo-oxygenase inhibition by non-steroidal anti-inflammatory agents

Theoretically a generalised reduction in renal prostanoid synthesis might improve CyA nephrotoxicity by inhibiting the enhanced production of TXA$_2$. The effect of CyA on the production of PGI$_2$ in the intact animal is less clear. If CyA also reduces PGI$_2$ production, a COO inhibitor will cause a further reduction such that the transplanted kidney may completely lose any ability to respond to vasoconstrictor influences by the production of vasodilator prostanoids. Loss of this protective response would be more damaging and unlikely to afford protection from CyA nephrotoxicity.
In other disease conditions characterised by impaired renal function, it is appreciated that renal blood flow is COO dependent, inhibition of this enzyme by NSAID's leading to further deterioration in function. There has been little work performed in transplant patients taking both CyA and NSAID's, however, Weinblatt et al (1987) investigated a group of patients with rheumatoid arthritis given 6mg/kg/d of CyA to study the response of their condition to this drug. 8 out of the 10 patients involved were already taking NSAID's and therefore the combined effect of these drugs was demonstrated. None of the patients studied had pre-existing renal disease as assessed by routine blood testing. All 10 patients developed a significant improvement in pain, swelling and stiffness of their joints over a 12 to 24 week treatment period. However, toxic effects including hypertension, occurred in 8 out of the 10 patients, necessitating withdrawal of CyA in 2 and dose reduction in the rest. Renal function, interpreted from serum creatinine levels only, showed deterioration from a mean value of 0.78mg/dl to 1.3mg/dl after 12 weeks of therapy (69μmol/l to 115μmol/l). In addition, three months after stopping CyA, serum creatinine levels had improved but not returned to pre-CyA values indicating possible permanent structural damage resulting from the combination of these drugs. Renal transplant recipients are generally advised against taking NSAID's as are all patients with a degree of renal dysfunction. Thus it seems that far from protecting against the constrictor influences of TXA₂ in CyA nephrotoxicity, NSAID's would in fact aggravate toxicity both functionally and structurally (Petric et al 1987).

2.10.ii Treatment with vasodilating prostanoids

More attention has been centred recently on the use of these substances in animal models of CyA nephrotoxicity where they appear to produce both
a structural and functional improvement in many of the features associated with CyA toxicity.

Gilas (1985) gave subcutaneous doses of dimethyl-PGE\(_2\) to rats prior to large doses of CyA. This substance improved renal function, survival and also renal morphology. Makowka et al (1985) described PGE\(_2\) as a "cytoprotective" agent after demonstrating a fall in serum creatinine and improvement in creatinine clearance in CyA toxic rats. PGE\(_2\) also decreased NAG enzymuria in CyA-toxic rats (Pomer et al 1985) who had undergone experimental renal transplantation, whilst Misoprostol (a PGE\(_1\) analogue) produced measurable increases in GFR and RBF in another rat experimental model (Paller 1988). PGE\(_2\) is the major vasodilatatory renal prostanoid produced by stimulated rat renal tissue. In all these models PGE\(_2\)-induced vasodilatation appears to counterbalance TXA\(_2\)-induced vasoconstriction. However, Ryffel et al (1986) suggested an alternative explanation for the beneficial effect demonstrated.

Treating spontaneously hypertensive rats with CyA and then with PGE\(_2\) caused BP to return to normal as did serum creatinine and GFR. Renal histology was also surprisingly normal in these animals who are prone to develop an exudative arteriolopathy in the presence of CyA. However, in combination with these features, it was also noted that peak levels of CyA were lower, producing a reduction in immunosuppressive activity, demonstrated by reappearance of the ability to produce a T-cell dependent antibody to sheep erythrocytes. In this model, therefore, PGE\(_2\) acted by reducing the bioavailability of CyA, possibly by reducing its intestinal absorption.

Prior to the introduction of Misoprostol, a PGE\(_1\) analogue, these substances were administered parenterally close to the required site of action. Large parenteral doses would be needed to reach adequate intra-
renal concentrations and to have the desired pharmacological effect. Side effects would be unavoidable and consequently very little work has been performed in man. Neumayer et al (1988) treated 65 renal allograft recipients taking CyA with Iloprost (a PGI₂ analogue), diltiazem (a calcium-channel blocker) or a combination of these two drugs. Graft function, measured as serum creatinine concentration and renal blood flow, were increased by 30% in those who received Iloprost alone or the drug combination. Diltiazem alone did not confer any beneficial effects.

In a Japanese study, patients with chronic damage in a renal allograft were given a combination of a PGI₂ analogue, an anti-platelet agent and an anticoagulant/anticomplement agent. This led to improved function and a decrease in both plasma TXB₂ and in the ratio of TXB₂/6kPGF₁α. It was concluded that platelets were the most important factor for renal deterioration in this model and that PGI₂ was protective. However, as several drugs were used in combination it is difficult to determine which contributed the maximum beneficial effect on renal function (Teraoka et al 1987).

2.10.iii Fish oils
Fish oils have also been used to modify the CyA-induced prostaglandin imbalance. The omega-3 fatty acids in fish oil serve as substrates for the cyclo-oxygenase pathway, competitively inhibiting the conversion of arachidonic acid. Any prostacyclin produced is in the PGI₃ form, an active vasodilator, however, TXA₃ produced by comparison, is inert. Fish oil therapy can improve vasospasm and cold intolerance in Raynaud's disease (Digiaccomo et al 1989) and it reduced TXB₂ synthesis both in renal cell culture and in rats treated with CyA. In the rat model, a significant increase in GFR was also recorded (Elzinga et al 1987a and
Further investigation of fish oil therapy in CyA nephrotoxicity is being undertaken.

2.10.iv Thromboxane Synthase Inhibition

Thromboxane $A_2$ was first recognised in 1969 as a substance which in bioassay contracted rabbit aorta, however, it was not named until 1975. The short half life of this substance in aqueous solution rendered synthesis difficult and therefore the chemical structure was not characterised until 1985. The slightly more stable endoperoxide precursors were characterised in the 1950's and synthetic analogues have been produced which serve as useful thromboxane-mimetics.

In 1977 it was noted that imidazole was an effective thromboxane synthase inhibitor (TSI) and several years later a series of selective synthase inhibitors were manufactured, the first being dazoxiben, a substituted form of imidazole (Halushka and Lefer 1987).

As CyA therapy enhances TXA$_2$ production in vitro and in vivo, inhibition of its production in the intact animal should allow the unopposed action of the vasodilatatory prostanoids PGI$_2$ and PGE$_2$, even if these are only produced in reduced quantities. Manipulation of the prostanoid balance should improve renal blood flow and hence function. A beneficial effect of TSI's has been demonstrated in several studies performed in animals and in man. In 1983, Tyler used dazoxiben and demonstrated decreased TXB$_2$ concentrations in addition to a concomitant increase in PGI$_2$ synthesis in man. TSI's inhibit TXA$_2$ production by both glomeruli and platelets as demonstrated by Lianos et al (1983) in a rat model of serum nephrotoxic nephritis. Reduced TXA$_2$ production could be detected in the supernatant and was associated with decreased adhesion when activated platelets were added to the system (Smith and Jubiz 1981). TSI's used
in vivo in similar models improve renal function. Scharschmidt et al (1983) demonstrated improved filtration fraction when treating patients with OKY-1581 (a TSI) whilst Petric and co-workers more recently (1987) noted a reduction in serum creatinine concentration in association with improved sodium clearance using a similar drug (U63557A) in CyA-toxic rats. In this latter model, improved renal function was associated with a reduction of TXB₂ levels to normal whilst PGE₂ concentrations, originally decreased by CyA therapy, increased, also returning to normal values.

The concomitant rise in the prostaglandins E₂ and I₂ in many models in response to treatment with TSI's has been widely reported (Smith and Jubiz 1981, MacGuire and Wallis 1983). Inhibition of TXA₂ synthesis leads to the accumulation of large quantities of endoperoxides, the precursors of all other prostaglandins. These substances are probably being utilised in the synthesis of vasodilatatory prostaglandins by endothelial and other cells and are therefore assisting further in correcting the CyA-induced imbalance in prostanoid production. The ability of endothelial cells to use platelet endoperoxides to synthesise other prostaglandins was demonstrated by the work of Marcus et al (1980). In an endothelial cell preparation COO activity was previously blocked by treatment with aspirin, the synthesis of all prostanoids being inhibited. When activated platelets synthesising TXA₂ were added to this system, detectable concentrations of PGI₂ were produced whilst at the same time, both thromboxane production and platelet aggregation were reduced. These findings suggest that platelet endoperoxides were being utilised by endothelial cells to make PGI₂. When a TSI was added to this system, excess endoperoxides accumulated and were used for the synthesis of even greater concentrations of PGI₂.
In a CyA-toxic animal, however, renal dysfunction is not completely reversed by thromboxane synthase inhibition. Endoperoxides are potent TXA₂ receptor agonists, producing vasoconstriction in their own right, in addition to being the precursors of all other prostanoids. It is the former action which Perico et al (1986a) believe to be responsible for incomplete improvement in GFR and lack of improvement in RBF demonstrated in CyA-toxic rats treated with UK 38485 (another TSI). Smeesters et al (1988) demonstrated improved short term renal function using a similar drug in CyA-nephrotoxic rats, however, structural defects associated with CyA nephrotoxicity persisted.

In conclusion elevated concentrations of endoperoxides accumulating from inhibition of the enzyme thromboxane synthase antagonise any beneficial effects resulting from the increased production of vasodilatatory prostanoids. Therefore, any improvement in renal function originally anticipated when treating CyA nephrotoxicity with a thromboxane synthase inhibitor will at most be incomplete.

2.10.v Thromboxane receptor antagonism

The other possible approach to ameliorating the effects of excess TXA₂ production induced by treatment with CyA is to use a selective TXA₂ receptor antagonist (TRA). These drugs do not affect the synthesis of individual vasodilatatory prostanoids. In addition this class of drugs has the advantage of blocking the action of prostaglandin endoperoxides which act on the same receptor. TXA₂ receptor antagonists suppress platelet function in addition to inhibiting TXA₂-induced vessel wall contraction (Oates et al 1988a and b).

Experimental work with these drugs has demonstrated a competitive mode of antagonism. Platelet-rich plasma from people treated with the TRA
BM13.177 showed a reduced tendency to aggregate *ex vivo*, whilst the *in vivo* bleeding time was also significantly prolonged (Gresele et al 1984). EP045 is another similar agent which competitively inhibited smooth muscle contraction induced by the TXA₂-mimetic, U46619, in dog, rabbit and guinea pig tissue (Jones et al 1982). Scharschmidt et al (1986) demonstrated that glomerular planar surface area could be reduced by a TXA₂ agonist and this effect was completely blocked by the presence of a thromboxane receptor antagonist.

Thromboxane receptor antagonists have not been widely used in clinical practice but in animal models of disease they prevent re-perfusion arrhythmias, reduce myocardial infarct size and inhibit platelet deposition on vascular grafts. These effects suggest a close relationship between prostanoid activity and the mobilisation of both intra- and extra-cellular ionised calcium. It has been suggested that TXA₂ may produce its effects via the release of Ca²⁺ ions from intra-cellular sources (Jaschonek and Muller 1988). A similar mechanism of action was also suggested by the work of Loutzenhiser and Epstein (1987) who showed that the TXA₂-mimetic, U44169, reduced GFR in the isolated perfused rat kidney, an effect which could be partially reversed by calcium-channel blockade.

In conclusion, evidence would suggest that an important mediator of the nephrotoxic effects of CyA therapy appears to be the excessive intra-renal production of thromboxane A₂. It was felt that concomitant treatment with a specific thromboxane receptor antagonist should block the vasoconstrictive effects of this prostanoid, improving renal blood flow and consequently improving long term renal function. Therefore, this work was undertaken to investigate the effect of treating a group of established renal transplant recipients with such a drug for a 3...
month period. Renal function was assessed accurately before and after the period of treatment.
CHAPTER 3
METHODS

3.1 Introduction

This study investigated renal function in a group of patients receiving cyclosporin A therapy. The majority of patients studied were renal transplant recipients having received their kidney transplant between 6 weeks and 24 months previously. An additional small group of subjects consisted of people suffering from an extensive and severe form of psoriasis or acute atopic eczema. Recent work has demonstrated that both of these conditions respond to cyclosporin A therapy (Heule et al 1988).

This study required reliable and accurate measurements of:-

i) Glomerular Filtration Rate (GFR) and Effective Renal Plasma Flow (ERPF)
ii) Cyclosporin A levels
iii) Prostaglandins TXB₂ and 6kPGF₁α in plasma and urine

A sensitive and reproducible technique for measuring renal function was required which could be repeated on several occasions in certain patients. As such GFR and ERPF were used to assess both the long term response to specific treatment as well as "natural" variations in function occurring over periods of several months. A modified form of these tests was also used which could be performed within a single day, demonstrating immediate and brief changes in function in response to a single stimulus.

The work described consisted of five individual studies performed with the assistance of the patients described. Each study required careful patient selection and included detailed explanation and discussion prior to recruitment. In order to complete each area of investigation within
the time available it was also necessary to overlap the study timetables.

All planning, recruitment and patient testing was performed solely by the investigator. All samples were collected in a routine and reproducible manner ready for laboratory assay. Those specimens required for the measurement of urinary and plasma prostanoids were collected into tubes which had been previously prepared in the Department of Surgery laboratories by the investigator. The individual samples were processed personally to minimise artefactual increases in prostanoid concentrations and then frozen until transportation to the Department of Chemical Pathology laboratories in Aberdeen for Radioimmunoassay.

Each measurement of GFR and ERPF took at least four-and-a-half hours to set up and perform. Blood samples were aspirated at accurately timed intervals throughout a 4-hour period. In total, over 180 individual measurements of GFR and ERPF were performed.

When investigating dynamic changes in renal function in response to an added stimulus, an infusion method of measuring GFR and ERPF was employed. Each took 8 hours to perform and required detailed preparation, supervision and sample handling. 18 of these studies were performed.

Wherever possible, these measurements of renal function were performed within working hours, however, a number were conducted at other times to avoid causing unnecessary inconvenience to patients who had recently returned to work after receiving a kidney transplant.

An acute dose of CyA associated with elevated measured blood levels of
drug is known to reduce renal blood flow and GFR in renal transplant recipients (Hauser et al 1988) and to reduce GFR alone in normal people (Weir et al 1989). Therefore each estimation of function performed required a concurrent measurement of blood CyA level.

Several explanations of the mechanism by which CyA adversely affects renal blood flow and function have been proposed. This study investigates further the theory concerned with excessive production of the vasoconstrictor prostanoid thromboxane A$_2$ (TXA$_2$) in response to CyA therapy. Contradictory evidence exists concerning the production of the counteracting vasodilatory prostanoid, prostacyclin (PGI$_2$), in man (Jorkasky et al 1987, Kho et al 1988). Both substances have very short half-lives in their active forms but are rapidly metabolised in vivo, to the stable products, thromboxane B$_2$ (TXB$_2$) and 6-keto prostaglandin F$_1$ alpha ($6kPGF_{1a}$) respectively.

A group of patients in this study received a TXA$_2$ receptor antagonist for a period of 3 months in an attempt to alleviate the nephrotoxic side effects of CyA. Measurements of urinary and plasma TXB$_2$ and $6kPGF_{1a}$ were performed, firstly to investigate the effect of CyA on the production of these substances and secondly to demonstrate any changes in their production which might result from the specific therapy.

3.1.(i) GFR and ERPF

In order to record function accurately, GFR and ERPF were measured using a technique which involved giving a single bolus injection containing two different radioactive isotopes. This method is simple both to perform and to repeat. It is preferable to conventional clearance techniques as timed samples of venous blood are required only, in contrast to the collection of both blood and urine samples. Collecting
urine samples also involves an additional problem of incomplete bladder emptying. This single injection isotope method is widely accepted as a reliable and reproducible technique for assessing renal function both in people with normal and deteriorating levels of function.

Standard radio-isotope doses were used which produce adequate plasma concentrations of radioactivity for detection and counting yet give a considerably lower radioactive dose to the patient than a routine chest X-ray. The isotope doses were measured and supplied by the Department of Medical Physics and Clinical Engineering at the Leicester Royal Infirmary, where the preparation and counting of plasma samples and calculation of results was also performed.

The clearance of the injected radioactive tracer, $^{51}$Cr ethylene diamine tetra-acetic acid ($^{51}$Cr EDTA) was used to measure GFR, whilst $^{125}$I orthoiodohippurate ($^{125}$I OIH) was used to measure ERPF. Both isotopes emit gamma irradiation which enables their presence in a sample of body fluid such as plasma or urine to be detected and the concentration measured easily with an automated gamma counter. The radiation emitted by each isotope differs in its energy level and therefore the concentration of each isotope in any sample can be measured separately whilst counting at each energy level simultaneously.

In the Department of Medical Physics at the Leicester Royal Infirmary, a detailed computer program has been written by several medical physicists. This applies the results obtained from the gamma counter to specific formulae used to calculate GFR and ERPF. From the results, the final values for GFR and ERPF are produced with ease and printed by the laboratory computer.

Renal function has been measured in Leicester by this technique for
approximately 7 years. Measurements of GFR are regularly performed within the district hospitals; the laboratory was responsible for the analysis of 192 tests during 1988. The measurement of ERPF is performed less frequently, being reserved more for use as a research tool, with the laboratory handling approximately 120 tests during the same year.

3.1.(ii) Cyclosporin A levels

All measurements of cyclosporin A (CyA) were performed in the laboratories of the Department of Surgery at the Leicester General Hospital. The Renal Transplant Unit was established in Leicester in 1977 and cyclosporin A therapy was introduced in 1983. This laboratory has measured CyA levels since 1984. Until 1986, all measurements of CyA were made by a radioimmunoassay (RIA) technique involving the use of a polyclonal non-specific antibody in a kit manufactured by Sandoz, (Basel, Switzerland - the manufacturers and suppliers of CyA). In Leicester, High Performance Liquid Chromatography (HPLC) has been used routinely to measure CyA since 1986 and results produced are both reliable and reproducible. This laboratory measures CyA levels for renal transplant patients from the local unit as well as providing a service for other departments. These include the department of haematology (for patients receiving bone marrow transplants) in addition to the departments of dermatology and ophthalmology who are assessing the efficacy of CyA in the non-transplant situation of diseases with a possible autoimmune basis. The laboratory performs two CyA measurements on each sample of blood submitted and the results are meaned to produce the final value. The total assay time for a single emergency sample is approximately 3 hours.

In order to compare the results from the many laboratories in the UK
using HPLC for the measurement of CyA levels, a national quality control is performed by Dr David Holt, Principal Biochemist at Guy's Hospital, London. Samples of human blood or plasma containing known CyA concentrations are distributed "blind". These are assayed by the routine technique in each centre and the results returned to the distributors. In this national quality control of CyA measurement, when laboratories are compared for the accuracy of their CyA measurements, the results from the laboratory at the Leicester General Hospital show a very close correlation with the values produced by the reference laboratory.

3.1.(iii) Prostaglandins

All prostaglandin measurements were performed in the Department of Clinical Biochemistry at Aberdeen Royal Infirmary, Scotland. Prostaglandin concentrations in specimens of urine and plasma were measured using Radioimmunoassay (RIA) kits manufactured by Amersham International, Amersham, Bucks. The laboratories in Aberdeen are experienced in RIA techniques which they have used since 1965. Prostanoid levels have been measured for research purposes by this method since 1984. This laboratory also has a particular interest in the mechanisms and effects of cyclosporin A nephrotoxicity. Approximately six prostanoid RIA's have been performed each year for the last 4 years in Aberdeen.

The kit supplied by Amersham contains all the necessary immunological reagents for the assay, including antigen labelled with the radioactive isotope $^{125}\text{I}$, specific antiserum and a quantity of the substance to be assayed from which standard solutions are made. These solutions of varying known concentrations are required to create a standard curve from which sample concentrations are obtained by interpolation.
Prior to the modern use of RIA kits, prostaglandins were measured by bioassay techniques which had the advantage of measuring biological activity of these compounds but were very inaccurate. Pieces of tissue examined in vitro produced large quantities of active prostanoids, however, this was not paralleled by an equivalent prostanoid formation in vivo. Bioassay is non-specific and cannot be used quantitatively to study prostaglandin turnover in man (Fitzgerald et al 1983).

Modern analytical approaches have been geared to a less direct measurement of stable but biologically inactive metabolites of the parent compounds.

Isotope dilution assays using the highly specific and highly sensitive Gas Chromatography-Mass Spectrometry (GC-MS), became the "gold standard" for the quantitation of prostanoids. Originally this procedure was cumbersome and required a range of expensive instruments, however, miniaturisation of these assays has simplified the technique (Catella et al 1986, Fitzgerald et al 1985).

RIA has the advantage of being relatively inexpensive and simple to perform only requiring access to a gamma counter and instruments found in most scientific laboratories. More recently antibodies of high specificity have been produced giving results approaching those of GC-MS whilst the use of the $^{125}$I isotope attached to the tracer has further increased the sensitivity of the RIA.

Urine samples contain large quantities of the prostanoids to be assayed and after extraction may require dilution. Plasma prostanoid levels, under normal conditions, are extremely low and concentration of samples may be required for detection.
Paired assays were performed on each sample and the results were averaged. As several assay runs were performed, 3 samples were taken from each run and assayed in the next consecutive run. If variation between assays exceeded 10%, the complete assay was repeated.

3.2 ISOTOPE MEASUREMENT OF RENAL FUNCTION

3.2.1 Principles of methodology for GFR and ERPF

For over 30 years the renal clearance of inulin has been recognised as the standard technique for the measurement of GFR (Smith 1955). Inulin is a polysaccharide extracted from the tuber of the dahlia plant and has a molecular weight of approximately 5000 Daltons. The use of this substance in the measurement of GFR depends upon its chemical nature and specifically the way it is handled within the nephron of the kidney. Inulin is metabolically inert, non-toxic and does not bind to plasma proteins. It has no detectable intrinsic effects on renal function and the renal clearance of this compound is independent of variations in plasma concentration. Solutions of differing inulin concentrations can be injected microscopically into various portions of the distal and proximal tubules of the rat kidney and are totally recovered in the urine from the injected kidney. None is absorbed and none appears in the peripheral blood or in urine collected from the contralateral kidney (Marsh and Frasier 1965).

The clearance of a substance is defined as the volume of plasma completely cleared of that substance in unit time (Gabriel 1985). In order to measure GFR, the substance used to measure clearance must satisfy the same criteria as inulin, being freely filtered at the glomerulus but neither resorbed nor secreted by the renal tubules. In this situation, clearance of that substance equals the glomerular
filtration rate. The rate of clearance must not be affected by any other substance and must remain constant despite changes in plasma levels and urine flow rate. Also, in order to measure GFR, this substance must be easily detectable and assayed in plasma and urine by simple, reliable and precise laboratory methods (Smith 1955).

No endogenous substance satisfies these criteria, although the 24-hour urinary clearance of creatinine is sometimes used as a rough estimate of GFR. However, creatinine production can be affected by many factors, including diet, exercise and lean body mass, whilst although being freely filtered in the glomerulus, it is also secreted within the distal tubule of the nephron. It has been clearly demonstrated that as the level of renal function deteriorates, creatinine secretion within the kidney increases and the relationship between true GFR and that measured as creatinine clearance does not remain constant (Bauer et al 1982).

Therefore in order to measure GFR accurately, an exogenous substance has to be introduced into the patient by intravenous injection. When using inulin, it is infused at a constant rate until a steady state is achieved with constant plasma levels. At this time the rate of renal excretion equals the rate of infusion. The bladder is emptied and all the urine produced over a timed period eg.1 hour \( (U_{vol} \text{ ml/min}) \) is collected. A venous blood sample is aspirated at the beginning and end of the collection period to calculate the mean plasma concentration during that period \( (P_{in} \text{ mg/ml}) \). An aliquot of the urine is assayed to obtain inulin concentration \( (U_{in} \text{ mg/ml}) \). Then using the UV/P formula (Austin et al 1921), GFR can be calculated:

\[
GFR = \frac{U_{in} \text{ (mg/ml)} \times U_{vol} \text{ (ml/min)}}{P_{in} \text{ (mg/ml)}} \text{ ml/min}
\]
This technique is usually performed by specialist personnel over a period of several hours, several clearances being recorded. It requires intravenous cannulation and constant infusion after a priming dose of inulin has been injected. Clearance periods require accurate timing and blood samples also need to be collected at the same time intervals. Unless the subject has a strong desire to pass urine at the required time, complete bladder emptying may not occur; ideally urine volumes should be collected via an indwelling urethral catheter. This is clearly a complex and invasive technique.

The inulin technique has been simplified over the years by the automated analysis of inulin in the laboratory and by attaching a gamma radiation-emitting tracer to the molecule to monitor bladder emptying without the need for catheterisation. However, the search for simpler methods has continued.

The use of radioactively-labelled isotopes attached to inert substances which satisfy Homer Smith's (1955) criteria for the measurement of GFR has led to the development of less complex methods of measuring both GFR and ERPF.

The two most widely used substances for estimation of GFR are $^{125}$I-labelled iothalamate and $^{51}$Cr-labelled ethylene diamine tetra-acetic acid (EDTA). The clearance of neither substance approximates exactly to that of inulin, the former slightly over-estimating and the latter slightly under-estimating true GFR. However, correction factors applied to the results obtained allow for these inconsistencies (Donker et al 1977, Heath et al 1968). These workers used constant infusion techniques with timed urine collections to measure renal clearance. More recently the use of a single intravenous bolus injection of isotope
has simplified the measurement of both GFR and ERPF further. This "plasma clearance" method assumes that once the dose of injected substance has reached a concentration equilibration with the extracellular fluid, the rate of disappearance of isotope from plasma is proportional to the rate of clearance of that substance by glomerular filtration (Duarte et al 1980b). Grossly oedematous patients do not reach equilibration rapidly because of their large extra-cellular fluid collections, hence this method is unsuitable for patients in those circumstances. No constant infusion is required and thus only a single intravenous cannula is required from which timed blood samples are aspirated. No urine samples are required at all which avoids errors produced by urinary tract obstruction, ureteric reflux, incontinence or inability to void on request.

After a bolus injection of $^{51}$Cr EDTA, the isotope used throughout this study, it is possible to calculate GFR from the concentration of isotope in a single, timed sample of blood only, taken exactly 3 hours after injection. However, several samples taken over a 4-hour period produce more reliable results (Constable et al 1979, 1985). It is not until GFR falls below 15ml/min that the sampling period needs to be extended beyond this time (Chantler and Barratt 1972).

Renal Plasma Flow is measured using a substance which is completely eliminated from the blood in a single passage through the kidney, by a combination of glomerular filtration and tubular secretion. Para-amino-hippurate (PAH) has been used widely as the standard substance for this purpose. It is non-toxic, minimally bound and does not penetrate the red cell. It is handled within the kidney by a combination of filtration and proximal tubular secretion and is relatively easy to assay in both blood and urine. As 8% of infused PAH bypasses functional
renal tissue, the term "effective renal plasma flow" (ERPF) is considered to be a more appropriate term to describe PAH clearance (Smith 1955). In an identical method to that described for the measurement of GFR, a constant infusion technique is used, with urine sampled from timed collections. The same formula is applied:

\[
ERPF = \frac{U_{PAH} \text{ (mg/ml)} \times U_{Vol} \text{ (ml/min)}}{P_{PAH} \text{ (mg/ml)}} \text{ ml/min}
\]

- \(U_{PAH}\) = urinary concentration of PAH (mg/ml)
- \(P_{PAH}\) = plasma concentration of PAH (mg/ml)
- \(U_{Vol}\) = urine volume (ml/min)

More recently, PAH has been replaced in the measurement of ERPF by radioactively-labelled substances. These compounds are easier to assay and can also be used to assess completeness of micturition. Ortho-iodohippurate (OIH) labelled with \(^{131}\text{I}\) is widely described in the measurement of ERPF and using the constant infusion technique, results compare well with those for PAH at values above 100ml/min (Donker et al 1977). This technique has been further simplified to a single intravenous bolus injection method which produces values which are slightly low when compared with PAH but demonstrate a reliable correlation (Wagoner et al 1964).

Throughout this study a single injection of \(^{125}\text{I}\) labelled OIH was used to measure ERPF. Again rapid equilibration was assumed followed by rapid clearance of this substance. Grossly oedematous subjects were unsuitable. The rate of disappearance of the \(^{125}\text{I}\) OIH from the plasma is proportional to the rate of renal clearance and hence the renal plasma flow.
In order to measure changes in GFR and ERPF in response to certain stimuli for example an oral protein load or a single dose of cyclosporin A, it was necessary to use a technique which would provide serial 1-hour measurements of GFR and ERPF over a study period with a maximum length of 8 hours. The single injection method is not suitable for this purpose as it produces a single value only for each test performed and thus an infusion method using the same radioactively-labelled substances was employed.

Each patient received the same standard bolus injection of each isotope given intravenously followed by a constant infusion, supplied by a small double-carriage infusion pump, of a dose previously calculated from known values of GFR and ERPF. In patients who had not undergone a recent functional assessment, level of function was estimated by comparison of serum creatinine concentrations with a range of results from similar transplant patients in whom GFR had been measured. ERPF was calculated from mean values for filtration fraction. Non-transplant patients with normal function in two kidneys, demonstrated by normal range serum creatinine levels, received a standard dose of each isotope. Therefore, the infusion dose was specifically tailored for the level of function in each patient such that plasma concentration remained constant throughout the period of investigation. To observe any variations in this which may have occurred, a plasma concentration of each isotope was measured from a blood sample collected both at the beginning and end of each clearance period and the two values averaged for the period under test.

Hourly urine collections have to be made during this study to measure the amount of each isotope cleared. The measured concentration of each isotope in each urine sample is directly related to GFR and ERPF over
FIG. 3.2.i Portable gamma radiation detector.
the previous hour. Variations in these concentrations are proportional to any changes in GFR and ERPF which may occur.

To reduce problems which may arise from a lack of desire to pass urine hourly, each patient received an oral loading dose of water ingested during the 60- or 90-minute period allowed for plasma isotope levels to equilibrate, at the onset of the study. This period of equilibration was longer than described by other workers but is preferable, especially when investigating people with reduced renal function (Wesson 1969). To maintain a good urine flow rate, bladder filling and hence desire to void with complete bladder emptying, the measured volume of urine passed during each hourly clearance period was replaced by drinking an equal volume of fluid. Completeness of bladder emptying was also confirmed by comparing counts recorded on a hand held gamma detector directed over the bladder area with those produced over the cardiac area. If the bladder was emptied completely, gamma activity was higher in the precordial area. (Fig 3.2.i)

3.2.ii Single injection isotope technique for the measurement of GFR and ERPF

On the day of study, patients attended after taking a light carbohydrate-based breakfast only, having omitted their morning dose of CyA.

Height and weight were measured accurately, recorded and later used in the calculation of Body Surface Area (BSA).

The patient rested supine on an hospital couch; after a 15-minute equilibration period, blood pressure was recorded by taking three measurements and calculating a mean value.

Using a brief period of tourniquet compression, a 21Fr gauge Butterfly
cannula (Abbott, Ireland) was inserted into a forearm vein and a 30ml sample of blood withdrawn. The intravenous cannula and attached tubing were flushed via the rubber bung with a solution containing 10IU of heparin per ml of sterile normal saline, prepared previously. The blood sample collected was divided into separately prepared and labelled tubes for the measurement of complete haematology, serum biochemistry and a CyA trough level in whole blood.

A second 21Fr gauge Butterfly cannula was inserted into a vein in the opposite forearm using a similar technique. This was also flushed with the heparinised-saline to prevent clotting within the tubing and also to ensure the correct positioning of the needle within the vein.

**Isotopes**

Single injection isotope clearances of renal function were performed using $^{51}$Cr EDTA to measure GFR and $^{125}$I OIH to measure ERPF. Syringes containing measured standard activities of each isotope were prepared and supplied by the Medical Physics Department at the Leicester Royal Infirmary. These were transported to the Leicester General Hospital in special lead-lined containers. (Fig 3.2.i) 5MBq of $^{51}$Cr EDTA in approximately 2ml was injected into the selected cannula, immediately following which a laboratory timer able to record minutes and seconds was started. This was immediately followed by an injection of 5MBq of $^{125}$I OIH, via the same cannula. The tubing was flushed with 10ml of the heparinised-saline solution and removed. This arm was not used again for venous sampling. If the patient had an arteriovenous fistula, used previously for haemodialysis access, that limb was used in preference for injection of the isotopes. A vein which was not in direct communication with the fistula was selected, therefore avoiding use of the fistula for repeated sampling.
FIG. 3.2.ii  Lead container, isotopes and minute timer used for estimation of single GFR and ERPF.
FIG. 3.2.iii Sampling venous blood via a Butterfly cannula.
Each sample of blood was aspirated from the remaining intravenous cannula after first removing and discarding 1ml of blood and saline from the tubing. A 5ml sample of blood was aspirated into a clean sterile syringe at 10, 20, 30, 40, 50, 60, 120, 180 and 240 minute intervals. (Fig 3.2.iii) Each was transferred to and stored in a tube containing lithium heparin anticoagulant, labelled with the date, name of the patient and the exact time of sampling, measured from the time of injection of isotopes. All samples were transported to the Department of Medical Physics for further analysis, at the end of the study. If any difficulty was encountered in adhering to the specified times, it was important to record accurately the times of any other samples collected. The empty isotope syringes were also returned to the same department in order to calculate exactly the injected dose of isotope.

Throughout the 4-hour study period the patient remained resting on the hospital couch. Tea and coffee were allowed but no meals other than a light snack or sandwich.

The morning dose of CyA was taken towards the end of the 4th hour. After the study, the cannula was removed and haemostasis was secured by local pressure. A small dressing was applied to the venepuncture site and the patient allowed home.

3.2.iii Infusion technique for measuring changes in GFR and ERPF in response to certain stimuli

Patients attended fasting on the morning of the study, having taken no solid food since the night before. A cup of tea or coffee was allowed for breakfast.

Patients already receiving CyA therapy had taken none since the evening dose of the previous day and none was taken during the study unless
specifically required, to exclude any undesirable, possible haemodynamic effect of this drug (Hauser et al 1988).

On arrival, weight and height were recorded. The patient then relaxed on an examination couch for 15 minutes prior to recording blood pressure which was measured as previously described.

The subject remained on the couch throughout the study which took 8 hours, only rising to pass urine at the stated intervals. The men passed urine standing, the women used a commode.

A 21Fr gauge Butterfly cannula was inserted in a forearm vein for sampling. A 20ml sample of blood was withdrawn for measurement of serum biochemistry, haematology and a trough CyA level measured in whole blood by HPLC. In patients with an arteriovenous fistula, this arm was used for sampling but vessels in direct communication with the fistula were avoided. After aspirating each sample, the cannula was flushed with a solution of normal saline containing heparin in a concentration of 10IU/ml.

An 18Fr gauge Venflon intravenous cannula (Viggo, Sweden) was inserted into a suitable vein on the opposite forearm and flushed with heparinised-saline.

Prepared priming doses of each isotope were supplied by the Department of Medical Physics at the Leicester Royal Infirmary. Individual priming doses of isotope were calculated from known recent values of GFR and ERPF and individual volume of distribution. The latter measurement was calculated by the computer program used in the measurement of GFR and ERPF. The priming doses were injected via the side-port of the Venflon cannula followed by 10ml of heparinised saline. A timer was started at
the mid-point of injection. Immediately after injection, an infusion
dose of each isotope diluted in 50ml of sterile normal saline was
delivered via a Y-cannula connecting two 50ml syringes mounted on an
infusion pump. The concentration of each isotope infused was calculated
for each patient from previous measurements of GFR and ERPF such that
plasma levels remained constant whilst the concentration of isotope
appearing in the urine was directly proportional to the GFR and ERPF
during the period studied.

Transplant patients who had not previously undergone measurement of GFR
and ERPF, had these values estimated by comparison of SeCr concentration
and 24-hour Cl\textsubscript{Cr} with measurements taken from other transplant patients
of similar body size. Dermatology patients with normal renal function
received standard doses of isotopes.

Oral hydration commenced with the subject taking a water load of 20ml/kg
body weight. An urine flow rate of between 2 and 10ml/min was
maintained throughout the study by replacing volumes passed with at
least an equal volume of water or diluted squash (Heath et al 1968).

Once the infusion had commenced, an equilibration period of at least 60
minutes was observed (Wesson 1969) after which time a 10ml sample of
blood was collected and the subject emptied his/her bladder. On this
occasion the volume of urine passed was discarded. A hand held gamma
detector was used to confirm the completeness of micturition.

The blood sample was transferred to a tube containing lithium heparin
anticoagulant for the later determination of isotope concentration in
plasma and when required, a second similar tube for estimation of CyA
whole blood concentration.
FIG. 3.2.iv Blood and urine samples collected during an infusion study.
Micturition was only encouraged when the patient had a strong desire to empty the bladder. If this desire was weak or absent, micturition would be incomplete. If the patient did not wish to pass urine at the designated time, it was postponed until the desire reappeared. With accurate timing of samples collected, variations in length of clearance intervals do not affect the results produced.

For determination of baseline GFR and ERPF, two 60-minute urine collections were made. The total volume of each was measured to the nearest 2ml using a measuring cylinder, recorded and a 10ml sample saved in a clean sealed tube for counting. Blood samples were collected at the beginning and end of each clearance period. (Fig 3.2.iv)

**Isotopes**

In renal transplant patients, GFR and ERPF were measured by $^{51}$Cr EDTA and $^{125}$I OIH clearances respectively. The required dose of each isotope was calculated from defining a system in which a constant volume of distribution is assumed. In this system, a bolus dose of isotope followed by a constant intravenous infusion maintains a steady plasma level when:

$$\text{infusion rate} = \text{renal clearance rate of that substance}.$$  

The loading doses were constant, each patient receiving 1MBq of $^{51}$Cr EDTA and 0.5MBq of $^{125}$I OIH. Infusion doses were individually calculated, each patient receiving between 1.19 and 4.2MBq of $^{51}$Cr EDTA and 1.7 and 6.0MBq of $^{125}$I OIH. The infusion doses were diluted in sterile normal saline to a volume of 50ml and were infused simultaneously at a rate of 5ml/hour.

Dermatology patients, had previously demonstrated normal renal function and received the same loading doses of 1MBq of $^{51}$Cr EDTA and 0.5MBq of
$^{125}$I OIH. Each also received an identical infusion dose of 8.4MBq of
$^{51}$Cr EDTA and 12.0MBq $^{125}$I OIH given diluted over a 10 hour period.

After performing baseline clearances an appropriate "stimulus" to renal
function was provided. After a suitable time interval further
clearances were measured in order to detect any changes in renal
function which may have resulted.

3.2.iv Quantitative analysis

MEASUREMENT OF GFR AND ERPF BY SINGLE INJECTION TECHNIQUE

1. Instrumentation

Philips PW4800 Automatic gamma counter
calibrated to detect gamma activity at 16-60 keV for $^{125}$I
270-370 keV for $^{51}$Cr

This machine uses a Thallium-activated sodium iodide crystal
scintillation detector and simultaneously measures gamma activity
at both energy levels. Each cassette holds 10 samples for
counting. (Fig 3.2.v)

Computer - Sirius 1
This calculates both GFR and ERPF from counts measured in the
timed-patient samples, using the radioactivity detected in the
standard solutions to estimate injected doses.
The two-compartment model described by Sapirstein et al (1955) is
used to calculate the final values.

Printer - Epson MX-100 III
Centrifuge- Beckman GP
Pipette - Volac R 880E 1000µl

101
FIG. 3.2. The Phillips PW4800 Automatic gamma counter.
2. Materials

(i) Radioactive isotopes supplied by Amersham International plc., UK.

\( ^{51}\text{Cr} \) EDTA for measuring GFR

Specific activity: 37-74MBq/mgCr

Radioactive concentration 3.7MBq/ml

\( ^{125}\text{I} \) ortho-iodo-hippuran for measuring ERPF

(containing <2% free iodide)

each ml of solution supplied contains 1% benzyl alcohol.

Specific activity: 0.37-1.85MBq/ml

Radioactive concentration 7.4-37MBq/ml

(ii) Standard solutions made up to contain

1. 2.5MBq \( ^{51}\text{Cr} \) EDTA in 250ml of sterile water

2. 2.5MBq \( ^{125}\text{I} \) OIH in 250ml of solution containing 20ml of a 0.5% sodium iodide solution in water.

3. Preparation of samples

A 1ml aliquot of fluid was aspirated from a previously sited 21 Fr gauge intravenous cannula to remove all traces of heparinised-saline. Following this, a 5ml sample of venous blood was aspirated smoothly into a clean syringe via a 19Fr gauge needle. The sample was immediately transferred to a tube containing lithium heparin anticoagulant-coated plastic beads and inverted several times to aid mixing. Each tube was labelled with the date, patient's name and the time at which the sample was collected, accurately measured from the mid-point of isotope injection. The samples were stored until the 4-hour test was completed and then transported to the site of counting. The two empty, capped syringes which had contained the original isotope doses for injection were also transported to the laboratory for counting. Patient height (metres) and weight (kilograms) were also recorded and supplied with the
samples.

All counting was performed in the Department of Medical Physics and Clinical Engineering at the Leicester Royal Infirmary.

The samples of blood were centrifuged at 2000 r.p.m. for 10 min following which a 2ml sample of plasma was separated by pipetting and placed in a carefully labelled, clean, disposable polystyrene counting tube.

4. Procedure

Labelled counting tubes containing the plasma samples, an empty tube to measure background radiation, and a 2ml sample of each standard solution were placed in a rack which was then inserted in the scintillation counter. The order of the tubes conformed to:

- position 1 Blank
- 2 $^{125}$I OIH standard
- 3 $^{51}$Cr EDTA standard
- 4 10min sample
- 5 20min sample etc.

The samples were counted on the Phillips automatic gamma counter, each being counted simultaneously for 20min at the two different energy levels to measure the activity of both $^{51}$Cr and $^{125}$I. A cross-over count of the high energy $^{51}$Cr into the $^{125}$I channel was also recorded and subsequently the percentage cross-over was subtracted from the $^{125}$I readings. The recorded number of counts per minute was automatically transmitted to the printer. The figures obtained were entered manually into the Sirius computer where analysis was performed using the program described.
5. Calculations

GFR from \(^{51}\text{Cr}\) EDTA

1 The injected dose of each isotope was calculated by subtracting the mass of the empty syringe from that of the full syringe prior to injection. (MP)

2 Timed samples were collected at 60, 120, 180 and 240 min after injection.

3 The program calculated the "true" activity of each isotope in each plasma sample by subtracting the counts from the blank or background tube made at each energy level. This subtraction was also made on the counts obtained from the standard solutions.

4 The counts for each sample were then converted to logarithmic values (y) and plotted against time after injection (x). The line produced was extrapolated backwards to produce an apparent plasma activity (Po) at zero time (To). From this value, an apparent volume of distribution was calculated from:

\[
V_d = \frac{MP \times STD \times \text{vol of std}}{\text{counts at To} \times \text{mass of std}}
\]

\[
V_d(\text{ml}) = \frac{MP(\text{mg}) \times STD (\text{cpm}) \times 250(\text{ml})}{Po(\text{cpm}) \times MS (\text{mg})}
\]

(Chantler and Barratt 1971).

MP = mass of patient's injection

STD = background corrected standard counts

MS = mass of standard

cpm = counts per minute

5 The line plotted consists of a bi-exponential curve; calculations were performed using the later straight line portion. The computer program applies linear regression analysis to calculate the slope of
"...and by applying a correction factor of 0.87" (0.9 in our program) "allows for the systematic difference between the slope clearance and UV/P..." (Chantler and Barratt 1971)

The half-time of the linear part of the slope was calculated from:

\[ T_{50} = \frac{60^* \times \log_e(2)}{gradient} \text{ min} \]

[*60 - times stored in decimal require correction to minutes]

Then GFR was calculated from:

\[ \text{GFR} = \frac{V_d \times \log_e(2) \times 0.9}{T_{50}} \text{ ml/min} \]

\[ \text{GFR} = \frac{V_d \times 0.693 \times 0.9}{T_{50}} \]

These calculations were all performed within the described computer program and a result for the non-standardised GFR was produced.

Each value is expressed for a standard body surface area of 1.73 m². In order to compare individual GFR's, individual BSA was calculated from the formula of Du Bois (1927):

\[ \text{BSA} = \frac{71.84 \times \text{Wt}^{0.425} \times \text{Ht}^{0.725}}{10,000} \text{ m}^2 \]

[Wt in kilograms, Ht in metres]

Standardised GFR was then calculated from:

\[ \text{GFR} = \frac{\text{GFR(pt)} \times 1.73}{\text{BSA(pt)}} \text{ ml/min/1.73m}^2 \]
GFR(pt) is uncorrected GFR
BSA(pt) is patient's body surface area

ERPF from $^{125}$I OIH

1 The injected dose of isotope, $^{125}$I OIH was calculated by subtracting the weight of the empty syringe from the full syringe prior to injection.

2 Background counts measured from the blank tube were again subtracted from sample counts and the standard solution.

3 Sample counts were also corrected for scatter from the $^{51}$Cr higher energy isotope.

4 Sample counts were measured at 10, 20, 30, 40, 50 and 60 minutes after injection of isotope.

5 Corrected counts per sample (y) were converted to logarithmic values and plotted against time (x). The curve produced was again bi-exponential, reflecting the two compartments of distribution described by Sapirstein et al (1955) following the injection of a bolus dose of creatinine into dogs.

Avendano and Novoa (1987) believe that compartment 1 approximates to the plasma volume and compartment 2 the extra-vascular fluid volume.

6 A tangent was constructed to the late portion of the curve (gradient = lambda 2). By a process of "curve stripping", the calculated second exponential, obtained from the late points on the curve, was subtracted from the whole curve leaving just the first exponential. From this a second tangent to the steeper, linear, early part of the curve was constructed with a different gradient (lambda 1). (Fig 3.2.vi) The intercept of each line on the y axis was recorded (A = intercept of lambda 1; B = intercept of lambda 2).
The slopes of the individual gradients were calculated from the half-life of the disappearance of isotope: (Wagoner et al 1964).

\[
\lambda_1 = \frac{0.693}{T_{1/2 \text{ 1st slope}}}
\]

\[
\lambda_2 = \frac{0.693}{T_{1/2 \text{ 2nd slope}}}
\]

Then:

\[
\text{ERPF} = \frac{\lambda_1 \times \lambda_2}{\text{STD} \times \text{MP} \times \text{dilution}} \times \frac{(\lambda_2 \times A) + (\lambda_1 \times B)}{\text{MS}}
\]

STD = counts from standard
MS = mass of standard
MP = mass of patient's injection

As with GFR, the results obtained were not corrected for a standard BSA of 1.73m² and therefore a similar correction was applied:
corrected ERPF = \text{ERPF} (\text{pt}) \times 1.73 \text{ ml/min/1.73m}^2 \text{BSA} (\text{pt})

GFR and ERPF from isotope infusion method

1 Calculation of isotope infusion dose

i Bolus dose

Standard bolus doses were given to all patients:

- $^{51}$Cr EDTA 1MBq
- $^{125}$I OIH 0.5MBq

ii Infusion dose

Unknown volume of dilution

For a patient with normal function estimated as a GFR of 140ml/min, 8.4MBq of $^{51}$Cr EDTA were supplied for a study of 10-hour duration.

For a patient with normal function estimated as an ERPF of 400ml/min, 12MBq of $^{125}$I OIH were supplied for a 10-hour study.

These doses assume an extra-cellular volume of 10,000ml and are the minimum doses which maintain a constant, detectable plasma concentration of each isotope in the presence of normal function.

The dose of each isotope infused was calculated as a proportion of the "normal" doses described above:

eg: For an estimated GFR of 40ml/min

\[
\text{Infusion dose of } ^{51}\text{Cr EDTA} = \frac{40}{140} \times 8.4 \text{ MBq} = 2.4 \text{ MBq}
\]

This dose was diluted to maintain a 10 hour infusion. No infusion ran for more than 8.5 hours in practice.

Volume of dilution known

In patients with a previously measured GFR and ERPF, the computer
program also calculated an individual value for the volume of dilution. This value was used to calculate the infusion dose of isotope for each patient, using the formula:

\[
\text{Infusion dose} = \frac{\text{Priming dose} \times (\text{GFR or ERPF}) \times 50 \times 60}{\text{Volume of dilution} \times 5}\]

50 = total infusion volume in ml
5 = infusion rate in ml/hour
60 = conversion from ml/hour to ml/min

This formula is derived from:

\[
\text{Plasma conc} = \frac{\text{Priming dose}}{\text{Dilution}}
\]

\[
\text{.. Amount infused} = \frac{\text{Priming dose}}{\text{Dilution}} \times \text{Clearance} \quad (A)
\]

\[
\text{ALSO}
\]

\[
\text{Amount infused} = \frac{\text{Infusion dose (MBq)}}{\text{Infusion volume (ml)}} \times \frac{\text{Infusion rate (ml/hr)}}{60} \times \frac{\text{MBq/min}}{
\]

\[
= \frac{\text{Infusion dose}}{50} \times \frac{5}{60} \times \frac{\text{MBq/min}}{(B)}
\]

\[
\text{..} \quad (A) = (B)
\]

\[
\frac{\text{Priming dose}}{\text{Dilution}} \times \text{Clearance} = \frac{\text{Infusion dose}}{50} \times \frac{5}{60} \times \frac{\text{MBq/min}}{}
\]

Rearranging:-

\[
\text{Infusion dose} = \frac{\text{Priming dose}}{\text{Dilution}} \times (\text{GFR or ERPF}) \times 60 \times 50 \times \frac{5}{\text{MBq}}.
\]

110
The infusion dose of each isotope was supplied in a volume of between 1 and 5ml. This volume was carefully transferred to individual 50ml syringes and diluted to make a total volume of 50ml with sterile normal saline. Each was infused with a dual infusion pump at a rate of 5ml/hr. The studies described were performed over periods of between 7.5 and 8.5 hours.

2 Preparation of samples

10ml samples of venous blood were collected throughout the infusion studies, a sample being taken at the beginning and end of each period of clearance measured. The blood was carefully transferred to a plastic tube containing lithium heparin-coated anticoagulant beads and inverted several times to aid mixing. Each sample was labelled with the date, name of the patient and the exact time interval since the bolus injections. The samples were stored upright overnight at a temperature of 4°C and were transferred to the Department of Medical Physics for counting on the following day.

The patient emptied his/her bladder at the end of each clearance period into a clean container. The total volume passed was recorded whilst a 10ml sample was collected and labelled with the name, date and time since injection of isotopes.

Each blood sample was centrifuged at 2000rpm for 5min after which if adequate plasma was available, a 3ml sample was accurately pipetted into a clean disposable polystyrene counting tube.

An equal volume (ie 3ml) of each urine sample was also accurately transferred into counting tubes.
3. Procedure

Instrumentation as above

Materials: standard solutions of the isotopes were not required for this method.

A 2ml $^{51}$Cr sample of approximately 20MBq was obtained from the 250ml stock solution.

Phillips PW4800 automatic gamma counter was used and the order of tubes in the counting cassettes was:

1. Blank - for background radiation
2. The $^{51}$Cr source to detect scatter from the $^{51}$Cr into the $^{125}$I channel.
3, 4, 5 etc. Blood samples followed by urine samples.

Each sample was counted for 20 minutes.

4. Calculations

Each sample represented on the printed output from the counter was identified. The counts produced were corrected firstly by subtracting background radiation recorded from the blank tube and secondly for scatter from the $^{51}$Cr into the $^{125}$I recording channel.

Plasma: The counts recorded for each isotope at the beginning and end of each 1-hour clearance period were added and divided by a factor of two to give a mean plasma concentration over the period of collection (P) cpm/ml.

Urine: The counts recorded per ml of urine (U) were multiplied by the total volume passed during that clearance period (V). This gives the total activity of the isotope cleared during that period.

To calculate the clearance during each period being investigated, the following formula was used:-
\[ \frac{U \times V}{P} \text{ ml/min} \]

(Austin 1921).

Again the values for GFR and ERPF required standardisation for a body surface area of 1.73m\(^2\).

3.2.v Sources of Error

1 Intra-assay variation
2 Personal technique
3 Variations in day to day GFR and ERPF

1 Intra-assay variation

In order to calculate the variation in the radio-isotope assay technique, 10 patients with a range of function were randomly selected. On the day of testing, instead of the usual 5ml sample of venous blood, a 15ml sample was aspirated. As this increased the time taken to collect the sample, the mid-point of the collection was recorded as the sample time. Three separate 5ml aliquots were placed in three tubes at each time and these were analysed in the laboratory as three distinct studies.

The three sets of results produced per patient were used to calculate assay variability.

Results

The GFR's of the patients selected ranged from 23.2ml/min to 68.17ml/min

ERPF's .. .. .. ranged from 115.73ml/min to 332.67ml/min

Coefficient of variation for each set of results for GFR assay

\[ 0.4 \text{ - } 4.27\% \] range

Mean CV for GFR = 4.01%
Coefficient of variation for ERPF assay

\[ 0.35 - 6.98\% \quad \text{range} \]

\[ \text{Mean CV for ERPF} = 2.54\% \]

There was no correlation between the level of renal function and the individually calculated coefficients of variation (using Spearman's ranked correlation coefficient).

These values do not include patient day to day variation or observer error.

2 Personal technique

All isotope studies were performed by one investigator, myself. The same method was repeated with ease in each patient and no technical problems arose from injection of isotope, sampling or timing of blood specimens.

3 Day to day variation

Within the limits of the work performed it was considered both unethical and, due to slow clearance of isotope in patients with poor renal function, technically difficult to perform repeated daily studies to ascertain this source of variation. However, a group of 6 patients with relatively good renal allograft function agreed to have their GFR and ERPF measured on three separate days during a 10 day period. The values produced were used to calculate day to day variation in these parameters.

Mean GFR of the patients studied = 52.7ml/min (Range 37.4 - 75.1ml/min)

Range of CV for individual GFR's

\[ = 5.01 - 12.82\% \quad (\text{Mean CV} = 8.06\%) \]

Mean ERPF of the patients studied = 273.8ml/min (Range 180-395ml/min)
Range of CV for individual ERPF's

= 8.69 - 19.49% (Mean CV = 11.5%)
3.3 HPLC MEASUREMENT OF CYCLOSPORIN A WHOLE BLOOD LEVELS

3.3.1 Principles of the methodology of CyA measurement

When CyA was first used therapeutically in 1976, an assay was not available to measure concentrations of drug in a blood or plasma sample. Therefore, the investigation of bioavailability, drug handling and compliance was also not possible. In 1981, Donatsch and colleagues introduced a Radioimmunoassay (RIA) technique for the measurement of CyA which could be performed on plasma specimens. The antibody used was raised in rabbits against the cyclosporin C molecule. However, it was soon realised that the antibody formed in this manner was not specific for the parent CyA molecule, tending also to bind CyA metabolites. It has been estimated that as little as 38% of a measured trough level of CyA, may result from the parent CyA molecule when using this type of polyclonal antibody in RIA (Kahan 1985). The contribution provided by "true" CyA is much greater when measuring peak levels but these are of little therapeutic value and are not routinely measured.

In long term monitoring of stable patients the concentration ratio of parent compound to metabolites remains fairly constant and the use of a non-specific assay is less critical, however, in the early post-transplant period this ratio may vary widely. In addition, the CyA molecule is thought to provide the major contribution to immunosuppressive activity (Freed et al 1987) and therefore a specific measurement of its concentration is indicated.

With the introduction of monoclonal antibodies by Milstein and Kohler in 1979 (Roitt 1984), antibodies specific for the parent molecule of CyA were soon synthesised. In 1987 Sandoz introduced a RIA for CyA which utilised such an antibody, hence avoiding the problems posed by cross-
reacting metabolites.

The measurement of CyA levels by High Performance Liquid Chromatography (HPLC) was introduced by Sawchuk and Cartier in 1981. Substances are separated on the basis of their polarity within the mobile phase. This separation results from the individual chemical and physical characteristics of different molecules under the conditions imposed. Quantitation of molecules of only slightly differing structure is therefore possible.

HPLC was the first specific assay available which provided a means of measuring "true" drug concentrations and for this reason, has always been used in Leicester for the measurement of CyA levels.

CyA is an hydrophobic, polar molecule which is known to exhibit a temperature- and time-dependent binding to erythrocytes, with over 50% of the drug bound at 37°C. This value increases markedly if the specimen is allowed to cool to room temperature. Erythrocytes appear to absorb CyA selectively by a mechanism which becomes enhanced at temperatures below 37°C (Niederberger et al 1983). In 1982, Kahan and co-workers introduced an extraction step which overcame the losses incurred from the measurement of plasma levels and so reduced the relative proportion of cross-reacting metabolites. Nowadays, centres which continue to use plasma samples use standardised separation protocols which minimise the influences of temperature and time.

Sawchuk and Cartier (1981) used whole blood samples in their HPLC assay of CyA concentrations and described a method which involved an extraction procedure. A general agreement now exists that whole blood measurements are to be preferred.
With the combination of extraction procedures for specimens of whole blood and specific monoclonal antibodies, the results from RIA now compare well with those for HPLC.

In our laboratory a modified form of the HPLC method described in 1984 by Varghese et al is used to assay CyA concentrations in trough samples of whole blood. An initial extraction of CyA is performed by mixing an organic solvent with a measured volume of anticoagulated blood. This dissolves the contained cyclosporins and other polar solutes and also precipitates proteins. This mixture is centrifuged and separated, the supernatant being subjected to purification by adsorption to a silica particle column. The column is washed with two different mixtures of solvent to remove contaminants. These mixtures contain different relative concentrations of polar (ethyl acetate) to non-polar (hexane) solvents. Within the column, cyclosporins and other polar compounds become attached to the silica particles. Non-polar compounds are firstly eluted out using a mixture with a greater relative proportion of hexane. This is followed by a mixture with a greater relative concentration of ethyl acetate. Finally, a cyclosporin fraction is eluted using pure, highly polar ethyl acetate. This is concentrated to dryness in a direct air stream and after reconstitution in 80% methanol, is suitable for estimation of CyA concentration by HPLC.

An aliquot of the final extract in solution is aspirated automatically by the sample processor from a closed container. 80% methanol is the liquid mobile phase within the automated chromatography column.

Chromatographic separation and quantitation relies upon the fact that substances of differing polarity pass through the silica column under the conditions imposed, at different speeds. The rate of separation
depends upon the strength of attachment of each substance to the particles within the column.

The high temperature within the chromatography column is essential to encourage rapid and dynamic release of the cyclosporin molecules from the silica particles. This renders them more easily detected within the spectrophotometer, producing separately identifiable narrow peaks on the moving chart. Each peak represents a detected compound or cyclosporin, the concentration of which is proportional to the area under the peak. Individual "Retention times" for CyA and CyD are dependent upon the type of column and mobile phase used in the chromatographic separation. These are the individual times which each cyclosporin takes to reach the measuring end of the column, calculated from the time of injection. It is necessary to ascertain these values for the specific column used for each assay. Retention times are measured for each assay run prior to patient sampling, during calibration. Using separate, pure, known concentrations of CyA and CyD, in our laboratory, the "Retention Time" for CyA is of the order of 7.4 to 9.5 min and that for CyD of 8.6 to 11.0 min (Fig 3.3.i). The purified cyclosporins are separated by passage through the column. The peak produced for each substance is identified and timed by comparison with a chromatogram produced by passage of the mobile phase alone (Fig 3.3.ii).

As it contains no aromatic ring structures, the cyclosporin molecule does not absorb light energy well in the ultra-violet (U-V) spectrum. However, maximum possible absorption is obtained with the spectrophotometer calibrated at 214nm.

By performing our routine, laboratory extraction technique with samples of blood to which a known concentration of $^3$H-labelled cyclosporin A had
### Calibration

<table>
<thead>
<tr>
<th>PEAK#</th>
<th>AMOUNT</th>
<th>RT</th>
<th>EXP RT</th>
<th>AREA</th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>500.0000</td>
<td>7.40</td>
<td></td>
<td>1950187 F</td>
<td>0.256300E0</td>
</tr>
<tr>
<td>2</td>
<td>500.0000</td>
<td>8.60</td>
<td></td>
<td>1934337 L</td>
<td>0.258400E0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PEAK#</th>
<th>RT</th>
<th>AMOUNT</th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.40</td>
<td>500.0000</td>
<td>0.256300E0</td>
</tr>
<tr>
<td>2</td>
<td>8.60</td>
<td>500.0000</td>
<td>0.258400E0</td>
</tr>
</tbody>
</table>

### Sample Position

- **Sample Position**: 01
- **Single Mode**: WISP Report

### Injection Details

- **Injection Volume**: 0020
- **Number of Injections**: 4
- **Injections Remaining**: 1
- **Run Time**: 00:11
- **Equilibration Delay**: 00:00
- **Non-Default Sys MSG's**: 6500-0030, 7048, 7508, 7601, 8601
- **WISP Codes Generated**: 63-01UL

### External Standard Quantitation

<table>
<thead>
<tr>
<th>PEAK#</th>
<th>AMOUNT</th>
<th>RT</th>
<th>EXP RT</th>
<th>AREA</th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>506.6920</td>
<td>7.44</td>
<td></td>
<td>1953544 F</td>
<td>0.256300E0</td>
</tr>
<tr>
<td>2</td>
<td>501.4050</td>
<td>8.60</td>
<td></td>
<td>1940422 L</td>
<td>0.258400E0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1002.0900</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Graphical Representation

**Figure 3.3.1** Calibration chromatogram for CyA and CyD demonstrating retention times.

- [7.44 min for CyA and 8.6 min for CyD]
FIG. 3.3.ii Program for calculation of CyA and CyD concentrations in the patient sample.
been added, the percentage loss during the extraction procedure has been calculated. In our laboratory, it has been demonstrated that 80% recovery of added CyA is possible, i.e. 20% of the CyA in the sample being lost during the extraction process. Thus the final concentration of CyA recorded from each patient sample, if uncorrected, will tend to underestimate the true whole-blood concentration. In every daily assay run, to correct for losses occurring during both extraction and HPLC, a known concentration of CyD is added to each original 1 ml sample of patient's blood and is subjected simultaneously to the extraction and chromatographic procedures. The final value produced for the concentration CyD on the printed results represents a percentage of the known added concentration. Consequently, the measured level of CyA in the sample will represent the same proportion of the "true" CyA and a corrected value is calculated to allow for losses throughout the assay.

A CyA whole-blood level between 150 and 400 ng/ml is generally accepted as within the therapeutic trough range for stable renal transplant patients receiving CyA as their major immunosuppressive agent. Although it is recognised that the CyA blood level is not an absolute indicator of the degree of immunosuppression or toxicity, most investigators agree that a level measured just prior to the next dose or "trough level" correlates best with the pharmacological response to the drug (Moyer et al 1986). Measuring levels at this time also eliminates some of the variability which can arise from differing rates of absorption.

3.3.ii Quantification of Cyclosporin A

1. Instrumentation (Fig 3.3.iii)

All instruments supplied by Millipore (Waters) UK.

Pump - Waters model 510
FIG. 3.3.iii  HPLC at the Leicester General Hospital.

Materials

- Calmodulin D supplied by Sandoz laboratories, Basel, Switzerland.
- Reagents supplied by Rmell Chemicals, UK (Loughborough).
- K8000, vinyl acetate, methanol
- Sep Pak silica cartridges - Millipore UK ltd
- Microsart - Limited-volume inserter
- Glass tubes
Injector - Waters Intelligent Sample Processor (WISP) 710B

Spectrophotometer - Lambda Max Model 481

The spectrophotometer is a variable wavelength model which is set to measure absorption at 214nm of U-V light. The maximum absorption of both CyA and CyD occurs at approximately 214 nm.

Column housing and temperature control unit - Waters Temperature Control Module

Data processing and printing - Waters Data Module 730

The complete HPLC system has a run time of 12 minutes per sample and is kept working day and night; our laboratory processes an average of 10,000 samples per year. The flow rate within the system is 0.5ml/min and the system is continuously sparged with helium to prevent the entry and accumulation of air which would affect the solvent/column equilibration. The retention time for CyA is approximately 8 min and that for CyD, 10min. As the column ages, retention times tend to decrease. The column is replaced after every 1000 injections.

Centrifuge - Heraeus Difuge

Agitator - Luckhams

Air Drier - SC3 sample concentrator

2. Materials

Cyclosporin D supplied by Sandoz laboratories, Basel, Switzerland.

Reagents supplied by Romil Chemicals, UK (Loughborough).

Hexane Ethyl acetate Methanol

Sep Pak silica cartridges - Millipore UK Ltd

Lichrospher RP Si columns - BDH, Poole, England.

Microsert - Limited-volume inserts

Glass tubes
Eppendorf Autodispenser pipette and plastic tips

3. Preparation of samples

A 4ml sample of blood was collected into a polypropylene tube containing lithium heparin anticoagulant-coated beads and inverted several times to ensure mixing. The samples were stored at room temperature and did not require immediate processing.

A 1ml aliquot of the sample was placed in a glass tube and each specimen was "spiked" with a known amount of cyclosporin D (500ng in 100µl). This was taken from a stock solution made previously by dissolving CyD in 80% methanol and stored at -4°C.

Ethyl acetate:hexane mixture (5ml of 20:80%) was added to this and the mixture shaken for 5 minutes at room temperature. Ethyl acetate helps to precipitate proteins and pigments from the blood sample. Following centrifugation at 5000rpm (4500g) for 5 minutes, the organic solvent supernatant was applied to a Sep Pak Silica cartridge which had been previously primed with 10ml of ethyl acetate followed by the 20:80 ethyl acetate: hexane mixture. The column was washed with a 10ml solution of the 20:80 ethyl acetate:hexane mixture, followed by a single wash with a 10ml solution of a 60:40 ethyl acetate:hexane mixture. Finally, the cyclosporins were eluted with 12ml of ethyl acetate alone. The elutant was collected in a clean glass tube and dried under an air stream at 70°C to produce an extract containing the added CyD, CyA and some impurities from the original sample. This was reconstituted in 100µl of the mobile phase, 80% aqueous methanol and transferred to HPLC glass inserts.

4. HPLC Procedure

The prepared methanol/cyclosporin solution was loaded into glass
microsert, limited-volume inserts which were themselves placed in spring loaded capped bottles. Each lid has a penetrable membrane through which the injection sample is taken. The capped bottles were loaded into a carousel which was placed in the WISP 710B injector. The model described can accept 48 or 96 samples in one run.

The WISP aspirated a 20µl sample from each glass microsert. This sample passed firstly through a Lichrospher Reverse Phase 8 encapped column containing silica particles of 5µm diameter, each with an attached 8 carbon chain. The mixture then passed into another identical column both columns being maintained at a critical temperature of 72°C by a temperature control unit. The separated compounds were quantified by passage via a spectrophotometer measuring absorption at 214nm.

The Data Module charted the results as a series of peaks on a continuously moving recording sheet (Fig 3.3.iv).
FIG 3.3.iv Flow chart for HPLC technique.
The Data Module also calculated the concentration of each substance present from the areas under the curves, by comparison with those produced by standard concentrations during calibration, and printed the results:

eg. for patient RP: (Fig 3.3.v)

\[
\begin{align*}
159.774 \text{ ng/ml} & \quad \text{CyA peak} \\
349.649 \text{ ng/ml} & \quad \text{CyD peak}
\end{align*}
\]

5. Calculation

In order to ascertain the concentration of CyA in the original sample of blood, the following calculation was performed:

\[
\frac{500}{ \text{Conc CyD} } \times \text{Conc CyA} = \text{true conc CyA in patient sample}
\]

eg.

\[
\frac{500}{349.6} \times 159.8 = 228.5 \text{ ng/ml of CyA}
\]
**Sample Position:** 80  
**Auto Mode**  
**WISP Report**

**Injection Volume:** 0020  
**Number of Injections:** 1  
**Injections Remaining:** 0  
**Run Time:** 00:14  
**Equilibration Delay:** 00:01  
**Non-Default Sys MSG's:** 6500-0030 PSI, 7096, 7601, 8601  
**WISP Codes Generated:** 63-01UL

**External Standard Quantitation**

<table>
<thead>
<tr>
<th>PEAK#</th>
<th>AMOUNT</th>
<th>RT</th>
<th>EXP RT</th>
<th>AREA</th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>159.77400</td>
<td>9.40</td>
<td>871177 F</td>
<td>0.183400E0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>349.64900</td>
<td>10.89</td>
<td>1926446 L</td>
<td>0.181500E0</td>
<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td>509.42300</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**FIG. 3.3** HPLC chromatogram from a patient sample (RP).
3.3.iii Analytical variation

1. Intra-assay variation

To calculate the coefficient of variation of our laboratory method, a 30ml sample of blood was collected from one patient. This sample was subjected to 10 separate analyses during the assay performed on a single day.

\[
\text{MEAN CyA LEVEL} = 182.80 \ (\text{range } 149 - 213) \ \text{ng/ml} \quad (n = 10)
\]
\[
\text{SD} = 17.29 \ \text{ng/ml}
\]
\[
\text{COEFFICIENT OF VARIATION} = 9.46\%
\]

2. Inter-assay variation

To calculate day to day variation of the assay method alone, further 1 ml aliquots of blood from a single-patient sample were analysed on different days during individual machine assay "runs". The results produced were subjected to similar analysis.

\[
\text{MEAN CyA LEVEL} = 155.3 \ (\text{range } 115 - 195) \ \text{ng/ml} \quad (7 \text{ days})
\]
\[
\text{SD} = 17.74 \ \text{ng/ml}
\]
\[
\text{COEFFICIENT OF VARIATION} = 11.4 \%
\]

3. National Quality Control Service

Since 1984 samples of unknown CyA content are circulated monthly from a central reference laboratory to UK and international hospital laboratories measuring CyA levels. The samples consist of human blood or plasma: some are taken from transplant patients, others are samples which have been spiked with known concentrations of CyA. The results are returned to the reference laboratory for processing. Results are circulated to the contributing centres on a "blind" basis.

For the period March 1988 to April 1989 (the period covering this work)
a median within assay CV of 2-10% was recorded for laboratories using
HPLC. (Range 1-59%)

Median CV's fell within the range 11 - 19% for the 25 transplant-patient

When our results measured from the distributed samples were plotted
against the true, known CyA concentrations, a good correlation was
produced

(r = 0.989, p<0.001 using Spearman's correlation)

with a plotted slope of +0.81.

In the Leicester Department of Surgery, HPLC measurement of CyA
concentrations compares well with results from the central reference
laboratory and also with other centres.
3.4 RADIOIMMUNOASSAY OF PROSTAGLANDINS

3.4.1 Principles of the methodology of Prostaglandin measurement

The prostaglandins are biologically active derivatives of arachidonic acid and are produced in small quantities close to their site of action. Arachidonic acid is released from cell membrane phospholipids by the action of the enzyme phospholipase A$_2$ and is further metabolised to the highly reactive endoperoxides, PGG$_2$ and PGH$_2$ by the action of cyclooxygenase. The endoperoxides are the immediate precursors of all prostaglandins, which are consequently chemically similar in structure but have widely differing physiological actions within the body.

This study was confined to the investigation and measurement of two prostanoids: thromboxane A$_2$ (TXA$_2$) and prostacyclin (PGI$_2$). Thromboxane A$_2$ is a powerful vasoconstrictor which also induces platelet aggregation and adhesion. It is produced mainly by platelets but also in certain tissues, in particular lung and kidney (Bunting et al 1983).

Prostacyclin is produced classically by vascular endothelium and has actions which are physiologically opposite to those of TXA$_2$. It is a vasodilator and is the most powerful inhibitor of platelet activation known (Whittle and Moncada 1983).

As both TXA$_2$ and PGI$_2$ are unstable substances with short half-lives, the production of each is more easily detected by measuring stable biologically inactive metabolites. Many enzymatically metabolised forms of both prostanoids have been identified. These include the 2,3-dinor and 11-dehydro compounds which can be detected and quantified by radioimmunoassay (RIA) using specific antibodies raised against each individual molecule. More complex methods of capillary-gas chromatography/negative-ion chemical ionisation mass spectrometry have
also been used. These enzymatic degradation products are thought to be produced mainly within the circulation and thus urinary concentrations are more indicative of systemic production of TXA₂ and PGI₂ (Catella et al 1986).

The compounds thromboxane B₂ (TXB₂) and 6-keto prostaglandin F₁ alpha (6kPGF₁α) are, however, non-enzymatic hydration products of TXA₂ and PGI₂ respectively. There is evidence to suggest that these particular metabolites are produced close to the local site of prostanoid synthesis, within the kidney. Therefore urinary concentrations of these substances may more closely reflect intra-renal synthesis of the parent compounds. However, evidence for this is inconclusive as urinary TXB₂ concentrations can also be elevated in syndromes associated with platelet activation (Catella et al 1986).

Unfortunately, when attempting to measure TXB₂ and 6kPGF₁α concentrations in plasma, the values obtained can be easily affected by the method of sample collection and storage. For example, prostacyclin release can be stimulated by trauma to the vascular endothelium. Ritter et al (1983) demonstrated that plasma levels could be elevated by a variety of stimuli including exercise, the venous "irritation" provided by an infusion of 10% dextrose solution, vein distension induced by infused normal saline and even minor trauma such as repeated sampling via a long indwelling intravenous cannula. It was concluded that a single venepuncture in a small forearm vein was least likely to affect plasma 6kPGF₁α concentration.

TXB₂ concentrations are similarly affected by the activation of platelets during blood sampling, which then continue to release TXA₂ during processing *ex vivo* (Ogletree 1987). This is a difficult problem
to avoid, circulating levels of TXA₂ are extremely low and platelet activation will cause relatively large artefactual increases in concentration. To avoid this, well described precautions were taken in the collection of plasma. A brief period of venous occlusion was used, hence avoiding traumatic venepuncture, which can be even more stimulating (Wilkinson et al. 1989). Each blood sample was aspirated into a polypropylene syringe, the surface of which was less likely to stimulate further platelet activation. The sample was immediately transferred to an ice-cooled polypropylene specimen tube containing two chemicals: EDTA anticoagulant to prevent clot formation and platelet activation, plus an adequate concentration of a non-steroidal anti-inflammatory agent (NSAID). These latter drugs are potent prostaglandin synthesis inhibitors and their addition prevents any further prostanoid formation within the sample.

Radioimmunoassay

RIA involves the competitive binding of radio-labelled and unlabelled antigen for a fixed number of binding sites on a specific antibody. This particular RIA involves competition between an unknown concentration of prostanoid in the patient-sample and a known quantity of the same compound bearing a radioactive label. Hence, the antigen is the particular prostanoid being quantified, whilst the binding protein is a specific antibody raised in rabbits.

In RIA, the radioactive label assists detection and quantitation of the antibody-antigen complex. ¹²⁵I is the radioactive isotope attached to the antigen supplied; it emits gamma irradiation of low energy detected in the range 16-60 keV.

If the patient sample contains a large quantity of the prostanoid to be
assayed, competition is great and less $^{125}$I labelled compound will become bound to the antibody. In this situation the radioactivity of the antigen-antibody complex will be low. Conversely, when the concentration of prostanoid in the patient sample is low the amount of radioactive tracer bound to the antibody will be high. Thus the concentration of the substance being measured in the patient sample is inversely proportional to the radioactivity of the complex.

Early RIA kits contained polyclonal antibody mixtures recognising several different areas of the particular molecule to be detected. This led to cross-reactivity with other chemically similar metabolites as well as with related substances, for example, the other prostaglandins. Modern RIA kits contain more specific antibodies which also have high affinity for the antigen and are therefore sensitive, being able to detect very low concentrations of individual metabolites.

The physiological sample containing the prostanoid to be quantified must first undergo an extraction procedure. This removes proteins and other contaminants which may interfere with the assay. Solid phase extraction was performed using standard manufactured columns containing silica particles. Once the sample to be assayed has entered the column, the contained prostanoid becomes reversibly attached to the particles. The column used for prostanoid extraction binds non-polar substances. Solvents of high polarity are first passed through the column dissolving and removing impurities of high polarity. Following this, graded solvents of lower polarity are passed through the column to remove substances of lower polarity. Finally, a non-polar solvent is passed which removes the prostanoid in a relatively pure form and this solution is saved. During this extraction process a known amount of a tritiated form of the prostanoid to be measured can be added to the column. At
the end of the process, the amount of this compound recovered in the eluted fluid is measured using a scintillation counter and the percentage extraction calculated. This value is used in final calculations to adjust for losses occurring from the patient samples during the extraction procedure.

The RIA kit supplies a measured quantity of the prostanoid being assayed. This is dissolved in a measured volume of solvent and further diluted to produce a range of solutions of known concentration. These are included in the assay to produce a standard curve from which sample concentrations can be interpreted.

The assay procedure involves adding a known amount of $^{125}$I radio-labelled prostanoid to equal volumes of samples and standard dilutions. To this is added an exact volume of the antiserum and this mixture is allowed to incubate.

In order to identify and separate the antibody-antigen complex, a second antibody is used. This is raised in donkeys against rabbit antibody and it also carries a pigment molecule attached to magnetizable polymer particles. After a further incubation period with the second antibody, the antibody-antigen complex is attracted to the bottom of the tube using a magnetic separator whilst the supernatant containing remaining free antigen is discarded. The amount of radioactivity in each tube is then measured using a gamma counter.

The known concentration of prostanoid in the standard dilutions is plotted semi-logarithmically, against percentage binding of the labelled tracer. This latter value is calculated from the radioactivity detected in the antigen-antibody complex. By reading off the radioactivity measured in the unknown patient samples, the concentration of prostanoid
in each can be deduced. This value is then corrected to allow for prostanoid lost during the extraction process.

If the sample being assayed contains a very high concentration of prostanoid, it can be diluted to produce radioactivity within the range of the standard dilutions. At the end of the assay, the final concentration of prostanoid is calculated by multiplying by the dilution factor.

3.4.ii Quantification of prostanoids

1. Instrumentation

Balance - Mettler ME22
Gamma counter - LKB Instruments, Ltd.
Refrigerator -20°C Sadia Airofreeze -80°C Kelvinator
Ultracold, Series 100, Manitowoc, Wis., USA.
Refrigerator/incubator - Zanussi
Vortex mixer
Polypropylene tubes - Sarstedt Ltd., Leicester.
Disposable polypropylene pipettes -
   automatic/tips - Sarstedt Ltd., Leicester.
Amerlex separator - Amersham International plc., UK.
pH meter - Horiba
Nitrogen drier
Columns for extraction - Bond Elut, Analytichem International.
Centrifuge - MSE.

2. Materials

di-potassium ethylene diamine tetra-acetic acid - BDH chemicals Ltd.,
   Poole, England.
Indomethacin, acid salt analytical grade - Sigma Chemical Company, St. Louis, Mo, USA.

Ethanol - analytical grade

Water - Double glass distilled and de-ionized

Reagents supplied by - BDH chemicals Ltd., Poole, England.

Citric acid (1M) Hexane
Petroleum ether Methyl formate

Radioimmunoassay kits - Amersham International plc., Amersham, Bucks, England. (Fig 3.4.i)

1. Thromboxane B₂ [¹²⁵I] assay system
   containing: TXB₂ standard, lyophilized
   Tracer, TXB₂ [¹²⁵I] iodo-tyrosine methylester, in ethanol:water (1:1).
   Rabbit Anti-TXB₂ antiserum, lyophilized.
   Assay buffer: Phosphate buffer concentrate, which on dilution yields 0.05M buffer, pH 7.3, containing 0.1% gelatin and 0.01% thimerosal.
   Amerlex-M donkey anti-rabbit serum coated on to magnetizable polymer particles, colour blue-green, 55ml. Ready to use.
   Results plotting sheet with semi-log graph paper.

2. 6-keto prostaglandin F₁α [¹²⁵I] assay system
   containing: 6-keto PGF₁α standard, lyophilized.
   Tracer, 6-keto PGF₁α [¹²⁵I] iodo-tyrosine methylester, in ethanol:water (1:1).
   Rabbit anti-6-keto PGF₁α, lyophilized.
   Assay buffer; Phosphate buffer concentrate which on
FIG. 3.4.1 Contents of the Amersham $^{125}$I RIA kit for 6-keto prostaglandin F_{1 alpha}.
dilution yields 0.05M buffer, pH 6.8, containing 0.05% bovine serum albumin.

Amerlex-M donkey anti-rabbit antibody as above.

Graph paper as above.

$^3$H (tritiated) TXB$_2$ and $^3$H 6kPGF$_{1a}$ - Amersham International plc., UK.

3. Preparation of samples

i) plasma

All samples of venous blood were collected with a brief period of tourniquet occlusion to minimise vessel trauma. A 21Fr gauge Butterfly cannula was inserted into a forearm vein and the sample was aspirated via its connecting 6cm length of plastic tubing. 6ml of venous blood was aspirated into a polypropylene syringe and rapidly transferred into a cooled polypropylene tube prepared previously and stored in a thermos flask of crushed ice. This tube contained 10µl of a 4mMol solution of di-potassium ethylene diamine tetra-acetic acid (EDTA) dissolved in deionized water. The tube also contained 10µl of a solution of the acid salt of indomethacin. This was originally prepared by dissolution in absolute ethanol, which was then allowed to evaporate off. The concentration used, contained 50µg of indomethacin, giving a final recommended concentration of less than 10µg per ml of blood. The tube was inverted several times to assist mixing and then centrifuged at 1500g for ten minutes. The plasma was separated with a polypropylene pipette and divided into two equal volumes of at least 1ml and each placed in a clean, ice-cooled empty polypropylene tube. Each tube was placed in crushed ice. These specimens of plasma were rapidly transferred to a low temperature refrigerator and stored at -80°C until the assay could be performed.
ii) urine

Each patient performed a 24-hour collection of urine at home on the day prior to attending for measurement of renal function. A 15ml aliquot of this was stored, frozen, in a clean plastic tube, at -20°C until assay could be performed.

iii) extraction

Both plasma and urine samples were allowed to thaw overnight in a refrigerator at 5°C. Prior to extraction, the urine samples were diluted in the ratio of 1:6 with normal saline, whilst the plasma samples did not require dilution. With this exception, the extraction procedure for TXB$_2$ and 6kPGF$_{1a}$ was identical both for urine and plasma specimens.

Solid phase extraction was performed using Bond Elut columns. The columns were first primed by the passage of 4ml of absolute ethanol followed by 4ml of water.

5μCi of tritiated prostanoid was added to 1ml of a biological sample to be extracted, to detect and measure losses of prostanoid which normally occur during this procedure.

180μl of absolute ethanol was added to 0.5ml of each sample. Each sample was acidified with 1M citric acid until a pH of between 3.0 and 4.0 was reached, measured with a pH meter using a micro-electrode. This sample was applied to the extraction column and its passage through encouraged by centrifugation. The columns were then washed twice, using 2ml of the polar solvent, 15% ethanol. Following this step, 4ml of 100% hexane, a solvent of lower polarity than ethanol, was used to wash the column.
Finally, 3ml of methyl formate, the non-polar solvent was passed through
the column, to remove the non-polar prostanoids being quantified. This
solution was dried under a stream of nitrogen leaving the prostanoid
extract.

At this stage, the content of the tritiated prostanoid added previously,
was measured with a scintillation counter. Percentage recovery of this
isotope was estimated and this value used to correct the concentrations
of sample prostanoids measured in the final calculations.

4. Radioimmunoassay procedure

i) preparation of assay buffer

The assay buffer provided was warmed to room temperature and transferred
into a 100ml graduated cylinder by repeated washing of its container
with de-ionized water. The final volume was adjusted to 75ml and mixed
thoroughly.

ii) $^{125}$I prostanoid tracer

The radioactively-labelled tracer supplied was diluted with 10ml of the
assay buffer and mixed using a vortex mixer. The resulting solution
contained approximately 74kBq of activity.

iii) antiserum

10ml of de-ionized water was added to the lyophilized antiserum and
mixed carefully to avoid the production of foam.

iv) preparation of standard concentrations

The standard solutions were prepared within one hour of performing the
assay as these prostanoids are rapidly adsorbed to the surface of the
containing tube. The lyophilized standard prostanoid supplied in the
RIA kit was reconstituted by adding exactly 1.0ml of de-ionized water.
This was mixed thoroughly to ensure complete dissolution. The resulting solution contained prostanoid in a concentration of 8ng per ml. This was allowed to warm to room temperature.

A range of eight standard concentrations was made. Eight polypropylene tubes were labelled with the concentrations: 3.1pg, 6.2pg, 12.5pg, 25pg, 50pg, 100pg, 200pg, and 400pg. 500µl of assay buffer was added to each tube. 500µl of the stock standard solution was added to the tube labelled "400pg" and mixed thoroughly. 500µl of this solution was then transferred into the tube labelled "200pg" and again mixed well. This procedure was repeated with each tube to produce successive halving of prostanoid concentrations, giving a range of standard concentrations from 3.1 to 400pg per ml.

v) Patient samples

The dried extract produced from each patient sample was reconstituted with 0.5ml of de-ionized water.

vi) Radioimmunoassay protocol

Day 1:

All reagents were allowed to warm to room temperature. Duplicate sets of polypropylene tubes were labelled for: Standards, Patient samples, Zero standard tubes, Total count tubes [TC] (contained labelled tracer, antiserum and buffer only), and Non-specific binding tubes [NSB] (contained labelled tracer and buffer alone).

A volume of assay buffer was added to each tube:

- 200µl to the standard and sample tubes
- 300µl to the zero standard tubes
- 400µl to the non-specific binding tubes (NSB)

-100µl of each diluted standard was added to the appropriately labelled
-100μl of each patient sample was added in duplicate to the appropriately labelled tubes and vortex mixed.

-100μl of 125I-labelled tracer was added to all tubes and vortex mixed.

-100μl of antiserum was added to all tubes except the NSB tubes.

-Total count (TC) tubes were capped ready for counting.

All tubes were again mixed using the vortex for 2-5 seconds then incubated for 16 hours at 5°C in a refrigerator.

Day 2:
The bottle containing the Amerlex donkey anti-rabbit antibody, which is blue-green in colour, was gently shaken and mixed. 500μl of this solution was added to every tube except the TC tubes. This mixture was again vortexed and then incubated for a further 10 minutes at room temperature.

Magnetic separation of antibody-antigen complexes from unbound tracer was performed using the Amerlex-M Separator. The tubes containing the mixture were placed in the rack in contact with the magnetised separator base and left for 15 minutes. After this time, keeping the tubes in contact with the separator base, the supernatant containing free unbound tracer antigen was poured off and discarded. The inverted tubes, still with associated separator, were placed on a pad of absorbent paper and allowed to drain for 5 minutes following which the rims were blotted on the paper to remove any remaining droplets.

The radioactivity of each tube was measured in the gamma counter for a period of 1 minute. A background count obtained from a blank tube was also included in the run.(B)
5 Calculations

Mean counts per tube were calculated from the duplicate values. The percentage binding of labelled tracer antigen occurring in each standard and sample was calculated from:

\[
\% \text{ bound} = \frac{(\text{Standard or sample cpm} - \text{NSB cpm})}{(\text{Bo cpm} - \text{NSB cpm})} \times 100
\]

\[\text{cpm} = \text{counts per minute}\]
\[\text{NSB} = \text{non-specific binding tube}\]
\[\text{Bo} = \text{binding of zero standard}\]

By plotting the percentage of tracer bound on the abscissa, (y axis), and \(\log_{10}\) of the prostanoid concentration of the standards on the ordinate, (x axis), a standard curve was constructed.

This plot was performed using a computer graphics program, which provided a calculated line of best fit. The results were also plotted by hand to ensure that single "wild" results did not greatly influence the shape of the standard curve.

Calculated percentage tracer bound in each patient sample was then used to read prostanoid concentration directly from the curve.

The concentration produced from the graph required correction for losses during the extraction procedure:
\[ \frac{^3\text{H PG added}}{^3\text{H PG recovered}} \times \text{PG conc of sample} = \text{true PG conc in sample} \]

This produced the prostanoid concentration in the plasma samples, whilst the values calculated from the urine samples also required multiplication by a factor of 6 to correct for dilution of the original samples.

3.4.iii Assay reliability

Three samples from each assay were subjected to repeat assay when the next run was performed. If the results of any of these samples varied by more than 10% from the original values, the complete assay run was repeated.

For this study, 4 assay runs were required. On one occasion, one of the three samples only, gave a value which showed more than 10% variation from the first result. The complete run was repeated; no differences exceeding 10% occurred on the second occasion.

The laboratory in Aberdeen quotes a coefficient of variation of approximately 6.3% for their measurement of prostanoids by radioimmunoassay using the Amersham \(^{125}\text{I}-\text{iodinated tracer kits.}\)

3.4.iv Specificity of antibody

Modern RIA kits contain antibodies with greater specificity for the compound to be quantified. However, some cross-reactivity still exists. In 1988(b), Benigni and colleagues attempted to measure the amount of cross-reactivity of a sensitive RIA for TXB\(_2\), using chromatographic separation and identification of extracted thromboxane-like immunoreactivity in biological samples. With this particular RIA, cross-reactivity for TXB\(_2\) was 100%, for 2,3-dinor TXB\(_2\) 10.5% and very low at
0.004% for 11,15-bis-dihydro TXB₂.

Amersham utilises highly specific polyclonal antibodies in their RIA kits and kindly agreed to investigate the cross-reactivity of the antibodies used with the 2,3-dinor derivatives of both TXB₂ and 6-keto PGF₁α. This work was performed after the termination of this research, at my request.

Amersham demonstrated that the antibody to TXB₂ had a 38.4% cross-reactivity with 2,3-dinor TXB₂ yet only a 2.5% cross-reactivity with 11-dehydro TXB₂. The 2,3-dinor derivative of 6-keto PGF₁α showed a slightly lower cross-reactivity of 23.8% with the antibody to 6-keto PGF₁α. Therefore, when concentrations of "TXB₂" and "6-keto PGF₁α" are described in both urine and plasma it is clear that these include a contribution from other metabolites of the respective parent compounds.
3.5 CLINICAL METHODS

All isotope measurements of renal function were performed following a light carbohydrate-based breakfast or a period of fasting.

On the day prior to isotope estimation of GFR and ERPF each patient performed a timed 24-hour urinary collection. This was used to calculate 24-hour clearance of creatinine, 24-hour protein excretion and in some instances was also used to measure daily excretion of prostaglandin metabolites.

Single injection and infusion isotope studies were performed with the patient resting on an hospital couch, rising only to pass urine when desired.

Prior to injection of isotopes and at all visits during the GR32191B drug trial, a venous blood sample was collected for estimation of routine haematology and biochemistry including renal, liver and bone function and a CyA trough whole-blood level (Fig 3.5.i). These samples were assayed in the appropriate laboratories at Leicester General Hospital by assay techniques which involved the use of computer based automatic analysers. The individual assay methods for both blood and urine samples are described in Appendix I.

Blood pressure was recorded at all visits and also hourly throughout all isotope infusion studies. On each occasion three separate measurements of BP were taken after the patient had rested for 15 minutes on the couch. A mean value for BP was calculated.

Renal vascular resistance was calculated from the formula:-

\[
\frac{MAP}{100} \times \frac{100}{ERPF} = RVR \quad \text{dynes sec/cm}^5
\]

(Curtis et al 1986a)
where \( \text{MAP} = \frac{(\text{Systolic} - \text{Diastolic})}{3} + \text{Diastolic} \) (mmHg)
<table>
<thead>
<tr>
<th>MEASUREMENT REQUIRED</th>
<th>Treatment period 3 months total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-treatment</td>
</tr>
<tr>
<td>Subject consent</td>
<td>X</td>
</tr>
<tr>
<td>Full clinical history &amp; physical examination</td>
<td>X</td>
</tr>
<tr>
<td>Blood Pressure</td>
<td>X</td>
</tr>
<tr>
<td>Blood for haematology</td>
<td>X</td>
</tr>
<tr>
<td>Blood for biochemistry</td>
<td>X</td>
</tr>
<tr>
<td>Blood for CyA trough</td>
<td>X</td>
</tr>
<tr>
<td>Urine sample taken for urinalysis</td>
<td>X</td>
</tr>
<tr>
<td>24 hr urine collection</td>
<td>X</td>
</tr>
<tr>
<td>GFR $^{51}$Cr EDTA</td>
<td>X</td>
</tr>
<tr>
<td>ERPF $^{125}$iodohippurate</td>
<td>X</td>
</tr>
<tr>
<td>Summary</td>
<td></td>
</tr>
</tbody>
</table>

*FIG. 3.5.1* Glaxo record book study plan.
3.5 PATIENTS

i) Long term trends in function in Leicester renal transplant recipients
44 patients completed the drug trial, having renal function assessed at
time 0 and 3 months later. (4 patients withdrew).
43 of these patients agreed to return for repeat testing of renal
function 6 months after commencing the trial.
31 patients returned at 12 months for further assessment of renal
function.

Only results from patients who had received placebo could be safely
assumed to represent general trends within the Leicester renal
transplant population. Therefore results from only
- 23 patients at 3 months
- 23 patients at 6 months and
- 15 patients at 12 months

could be utilised in the final analyses.

ii) Is serum creatinine a reliable indicator of renal transplant
function?
All 48 sets of baseline results and 43 sets measured at 3 months were
used to investigate the relationships between commonly accepted
parameters of renal function and the more accurate isotope measurements
of GFR and ERPF.

iii) The effect of the thromboxane receptor antagonist ,GR32191B, on
renal transplant function
49 patients were recruited to this trial and each gave written informed
consent; 48 commenced the trial.
44 patients completed the three-month period of treatment.
iv) The acute effect of Cyclosporin A upon renal function

The acute effect of CyA was investigated in two groups of patients. It was hoped that after demonstrating an acute haemodynamic effect, attempts could be made to modify the changes produced in this model, using the TXA$_2$ receptor antagonist, GR32191B.

Firstly, the effect of a "normal" therapeutic CyA dose was investigated in 4 stable renal transplant recipients who were not taking nifedipine and were not therefore involved in other studies.

Following this, the effect of a large, single, oral, loading dose of CyA on renal haemodynamics was investigated in 3 patients with severe psoriasis and 1 with severe atopic eczema.

v) Renal Functional Reserve in patients receiving CyA

This was investigated in 9 of the patients recruited to the GR32191B drug trial. The individual studies were performed between 6 and 12 months after completing the drug trial. Of these patients, 5 had received active drug and 4 placebo.

All studies were performed with the approval of the local ethical committee.

(Fig 3.6.i)
FIG. 3.6.4
Patient undergoing isotope infusion study.
3.7 STATISTICAL ANALYSIS

All data were analysed using Oxstat V computer program on a personal computer IBM PS/2 Model 30.

Results are expressed as mean values in tables, with +/- one standard deviation quoted in parentheses in the text. Occasionally, a mean value differed widely from the statistical median; in those situations, the median value has been quoted in addition for completeness.

In view of this occasional finding, the range of results produced from the patient population investigated cannot be assumed to be normally distributed. Consequently, non-parametric statistical analysis has been performed throughout.

To investigate comparisons between randomly selected groups prior to manipulation with either drug or placebo, a Mann Whitney-U test was used. This is the non-parametric equivalent of the unpaired t-test.

The Wilcoxon ranked sign test was used to compare changes in values recorded before and after a particular manipulation, for example, giving a large oral protein stimulus.

Correlations were performed where necessary using the Spearman ranked correlation coefficient for non-parametric data.

In all cases significance was assumed at the 5% level with \( p = 0.05 \). With greater statistical significance a \( p \) value is given.
RESULTS

4.1 LONG TERM TRENDS IN FUNCTION IN LEICESTER RENAL TRANSPLANT RECIPIENTS

INTRODUCTION

Renal transplant function was examined both retrospectively and prospectively in a group of patients who were participating in a placebo-controlled drug trial. Only results from patients who received placebo were used for analysis. Investigations were performed to discover if the Leicester renal transplant population demonstrated changes in function consistent with chronic CyA nephrotoxicity and might therefore benefit from a drug which would arrest or reverse this deterioration.

Firstly, data from all patients transplanted since the first use of CyA in 1983 were reviewed and analysed to discover the local experience with this immunosuppressive agent. Particular attention was paid to graft survival and sequential function which to date had only been monitored by regular measurement of serum creatinine (SeCr) concentration.

Secondly, all patients who participated in the GR32191B trial had received their transplant during or after 1986. The majority of these people agreed to have repeated detailed assessment of renal function 6 and 12 months after their initial investigations. Data were therefore collected from patients receiving both active drug and placebo. As will be discussed, GR32191B produced significant changes in renal function and therefore results from patients taking active drug were excluded from this particular study. The study code was not available until late in 1989; it was therefore not possible to investigate only those patients receiving placebo.
RESULTS

4.1.1 Retrospective investigation - patients receiving CyA

Between January 1984 and the end of December 1988, 214 patients received a renal allograft in the Leicester Transplant Unit; all but 2 were from cadaveric donors. Of these patients, 205 received CyA immunosuppression in combination with prednisolone and in a small number of patients as part of a triple therapy regimen with azathioprine. By the end of 1989, 69 of the 205 grafts had ceased to work; 12 of these patients had died of unrelated causes with a functioning graft in situ. Of the 57 remaining failed allografts, 14 patients died as a result of graft failure and its associated complications.

The results of the retrospective assessment were discussed in more detail in section 1.3. Briefly, however, 1-year graft survival rates were improved in 1984 and 1986 but then demonstrated a deteriorating trend in 1987 and 1988 (Fig 4.1.i).

Examination of SeCr concentrations in grafts still functioning at the end of 1989, showed a definite trend for SeCr levels to rise slowly with time after transplantation. This progressive deterioration, was particularly demonstrated in those patients with relatively good early function (Fig 4.1 ii). This way of representing the data unfortunately selects for patients with better renal function, excluding the effects of grafts with early poor function which have failed in the interim period. However, progressive dysfunction is clearly demonstrated.

It is of interest that patients transplanted in 1986 have the best early and continuing renal function as demonstrated by SeCr measurements, in addition to relatively good percentage survival at the end of the 3rd year.
FIG. 4.1.1 Percentage graft survival per year following transplantation.
FIG. 4.1.ii  Mean serum creatinine concentration per year following transplantation for kidneys still functioning in 1989.
Both immunological matching and treatment protocols remained unchanged in Leicester between 1984 and 1989 and therefore are unlikely to be responsible for the differing levels of renal function demonstrated between patients transplanted in different years. On further investigation, one possible explanation for the better function in kidneys transplanted during 1986, was the overall younger age of donor organs available and therefore accepted for transplantation during that year (Table 4.1.1). Better continuing renal function was also demonstrated by the selected patients who underwent a more rigorous assessment (Appendix II). Clearly both the median and mean age of donor kidneys has increased over the last 3 years, a factor which has probably contributed significantly to deterioration in both graft function and overall long term graft survival. These two features may also be intimately related to the sensitivity of the transplanted kidney and to the nephrotoxic effects of CyA.

<table>
<thead>
<tr>
<th>YEAR of TRANSPLANT</th>
<th>MEDIAN DONOR AGE</th>
<th>% DONORS &gt; 40YRS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1984</td>
<td>32</td>
<td>35.6</td>
</tr>
<tr>
<td>1985</td>
<td>28.5</td>
<td>38.9</td>
</tr>
<tr>
<td>1986</td>
<td>30</td>
<td>33.3</td>
</tr>
<tr>
<td>1987</td>
<td>39</td>
<td>42.2</td>
</tr>
<tr>
<td>1988</td>
<td>41.5</td>
<td>55.6</td>
</tr>
</tbody>
</table>

Table 4.1.1 Trends in donor age during CyA use in Leicester.
4.1.ii Prospective study - patients transplanted during 1986-1988

One criterion for recruitment to the GR32191B trial was a SeCr concentration not exceeding 300 μmol/l. Consequently, the patients investigated are not truly representative of the total renal transplant population in Leicester. However, the results obtained can be used to demonstrate local trends in renal function and hence gauge the success of renal transplantation in Leicester using CyA immunotherapy. These trends may be more apparent in patients who originally demonstrated good renal function.

The patients studied can be conveniently divided into 3 groups having received their transplants in 1986, 1987 and 1988 respectively (Table 4.1.ii). Each patient, when agreeable, was subjected to 3 assessments of renal function, separated by two 6-month intervals (Appendix III).

<table>
<thead>
<tr>
<th>YEAR OF TRANSPLANT</th>
<th>STUDIED DURING</th>
<th>MEAN/MED DONOR AGE</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A 1986</td>
<td>3rd year</td>
<td>29 / 24</td>
<td>n=9</td>
</tr>
<tr>
<td>Group B 1987</td>
<td>2nd year</td>
<td>35.2 / 34.5</td>
<td>n=7</td>
</tr>
<tr>
<td>Group C 1988</td>
<td>1st year</td>
<td>40.9 / 45</td>
<td>n=7</td>
</tr>
</tbody>
</table>

*Table 4.1.ii* Groups investigated, defined by year of transplant.

Median values were used in the plots as mean values tended to be skewed by particularly extreme measurements in these relatively small groups. Fig 4.1.iii illustrates the changes measured in median values of GFR and ERPF demonstrated in the groups described. Fig 4.1.iv similarly shows the values of SeCr and $\text{Cl}_\text{Cr}$ measured at the same times, whilst Fig 4.1.v shows RVR calculated from measured blood pressure. The other figure, 4.1[vi illustrates changes in CyA dosage and whole-blood trough level
which coincided with the changes in renal function recorded.

Care must be taken when interpreting these plots. For example, when examining median values for GFR or SeCr at the same point in time, comparing Group A with Group C, it must be remembered that the measurements in these two groups are temporally separated by a period of 2 years. However, the apparently better function demonstrated throughout by Group A (transplanted in 1986) would appear to be a true difference. As already shown (Fig 4.1.ii), SeCr levels for all patients transplanted in 1986 were markedly better at the end of the 1st and 2nd year than comparable values for patients transplanted in either 1987 or 1988.

The patients who were transplanted in 1986 also demonstrated relatively stable renal function over the period of investigation, particularly in terms of GFR, SeCr and RVR.
FIG. 4.1.iii Trends in median values of GFR and ERPF during the 12 month study period.
FIG. 4.1.iv Trends in median values of serum creatinine and creatinine clearance.
FIG. 4.1.v  Trends in median values of RVR.
In contrast, patients transplanted during 1988 demonstrated a more rapid rate of functional deterioration throughout the period of study which was even more marked in the second 6 months. In an attempt to combat this rapid deterioration in function, it can be seen that CyA doses were rapidly lowered; this manoeuvre was also reflected in falling whole-blood trough concentrations of CyA, however, no improvement in function resulted.
FIG. 4.1.vi CyA dosages and whole blood trough levels.
CONCLUSION

Patients in the Leicester Renal Transplant Unit clearly demonstrate a chronic progressive loss of kidney allograft function consistent with the recorded effects of CyA nephrotoxicity. Despite improvement in graft survival in the early years of experience with CyA, the more recent policy of using organs from older donors has probably contributed to the loss of these early advantages.

More detailed investigation of a number of patients transplanted between 1986 and the end of 1988 demonstrated a deterioration in early transplant function recorded since 1986. This finding was noted in addition to a continuous progressive deterioration in renal function, demonstrated in most patients from the time of transplantation.

Therefore, it was felt that these patients would benefit from a drug which might counteract the nephrotoxic effects of CyA. A specific thromboxane receptor antagonist was introduced on this basis.
INTRODUCTION

In the majority of renal transplant units, SeCr is monitored and interpreted as a reliable indicator, demonstrating changes in renal function. Sometimes only single values are measured at infrequent intervals.

Measurements of SeCr were recorded in a group of transplant recipients and several mathematical manipulations of these values were performed. The results obtained were compared with a more accurate determination of true GFR measured by an isotope clearance technique.

RESULTS

48 patients had their renal function assessed at the beginning of the study.

44 of these patients returned for repeat assessment 3 months later. Changes recorded in SeCr concentration were compared with changes in GFR occurring over the same time period.

15 women and 33 men, with a mean age of 43.2 years (SD 14.37yr) participated at the onset of the study.

The baseline mean value for GFR recorded in these patients was 45.2ml/min (SD 12.3ml/min) with a range of between 22.5 to 73.9ml/min. Mean SeCr concentration was 152.6μmol/l (SD 54.2μmol/l), again with a wide range from 76 to 306μmol/l.

It has been suggested that the Gault and Cockcroft formula is a more accurate method of using SeCr concentration to monitor renal transplant function (Cockcroft and Gault 1976, Myara et al 1979), as body weight is
included in the calculation. In this study the Gault and Cockcroft formula was used to calculate a value for "GFR" from the SeCr measurements recorded.

\[
\text{Gault & Cockcroft "GFR"} = \frac{(140 - \text{age}) \times (\text{body weight in kg})}{0.814 \times \text{SeCr in } \mu\text{mol/l}} \text{ ml/min}
\]

[females 15% less]

Data collected from the two studies are shown in table 4.2.i

<table>
<thead>
<tr>
<th></th>
<th>1st mean +/-SD</th>
<th>Range</th>
<th>2nd mean +/-SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>SeCr</td>
<td>152.6 (54.2)</td>
<td>76-306</td>
<td>154.4 (56.7)</td>
<td>74-295</td>
</tr>
<tr>
<td>ClCr</td>
<td>59.1 (23.2)</td>
<td>24-118</td>
<td>60.1 (31.0)</td>
<td>15-173</td>
</tr>
<tr>
<td>true GFR</td>
<td>45.2 (12.3)</td>
<td>22.5-73.9</td>
<td>44.1 (16.2)</td>
<td>22-84.3</td>
</tr>
<tr>
<td>Gault &amp; Cockcroft &quot;GFR&quot;</td>
<td>55.4 (17.9)</td>
<td>27.3-98.1</td>
<td>56.7 (21.0)</td>
<td>23.3-117</td>
</tr>
</tbody>
</table>

**Table 4.2.i** Measurements of renal function separated by a 3-month interval.

When the first set of data for all patients was compared with results obtained 3 months later, no statistical differences could be detected. Mean values and ranges measured remained very similar over this short period of observation. However, individual measurements did not necessarily follow the same trend.

Plotting 1st GFR against 1st SeCr concentration produced the exponential type curve characteristically described (de Wardener 1985). As renal allograft recipients do not demonstrate levels of GFR approaching the normal range for patients with two functioning kidneys, the number of points falling above 60ml/min on the x axis is small (Fig 4.2.i).
FIG. A.2.1 First serum creatinine v first isotope measurement of GFR.
Examining the data more closely, the plot described was divided about the mean GFR of 45.2 ml/min into two roughly equal sized groups of patients. From this manipulation of the data, it can be seen that those patients with a GFR less than 45 ml/min demonstrated a close correlation between SeCr concentration and the isotopically measured GFR ($r = -0.52$, $p = 0.007$, Fig 4.2.ii). Patients with better renal function and a GFR above 45 ml/min demonstrated no relationship between these two variables (Fig 4.2.iii, $r = -0.31$, $p = 0.151$).
Correlation = -0.52
p = 0.007

FIG. 4.2.ii GFR v serum creatinine in all patients with a GFR < 45ml/min.
Correlation = -0.31
p = 0.151NS

FIG. 4.2.iii  GFR v serum creatinine in all patients with a GFR > 45ml/min.
Creatinine clearance measured from a 24-hour urine collection is considered by some to provide a more reliable indicator of true GFR than SeCr concentration. However, in the presence of renal dysfunction, Cl\textsubscript{Cr} deviates further from true GFR than the value of 10-20% quoted for people with normal function. Values for Cl\textsubscript{Cr} in the transplant patients varied widely when compared with measurements of true GFR, no constant relationship between the two was demonstrated. In these patients, Cl\textsubscript{Cr} overestimated true GFR by a mean value of +32.28%, whilst a very wide range of variation occurred from -27.57% to +188.89%. No correlation existed between the degree of over- or under-estimation of GFR by Cl\textsubscript{Cr} and the corresponding value of true GFR (r = -0.044, p = 0.77). The correlation between Cl\textsubscript{Cr} and true GFR was significant (r = 0.64, p < 0.001, Fig 4.2.iv). Again, patients with a lower level of function (GFR < 45ml/min) demonstrated a much closer relationship (r = 0.66, p < 0.001). At values of GFR above this level, no statistical relationship between these two variables could be demonstrated (r = 0.36, p = 0.086 ns).

The plot of 1/SeCr against GFR (Fig 4.2.v) has been proposed as a useful indicator of level of renal function, particularly in patients with chronic renal failure. Some authors believe the individual slope for a patient indicates the rate of renal deterioration (Mitch et al 1976, Rutherford et al 1977). However, it is naive to assume that renal function deteriorates at a constant rate (Levey et al 1988).
FIG. 4.2.iv  GFR v creatinine clearance.
Correlation = 0.698
p < 0.001

FIG. 4.2.v GFR v the reciprocal of serum creatinine concentration.
The measurements of GFR and ERPF recorded from the isotope clearances were standardised to allow for variations in body surface area (BSA). The individual BSA for any patient was calculated from weight and height. The results obtained were used to calculate clearance values for an average body surface area of 1.73m$^2$. Some nephrologists believe that SeCr levels should also be adjusted to allow for variations in body size in order to bear a closer relationship to lean-body mass.

The Gault and Cockcroft formula was originally described to calculate a value for $C_{\text{Cr}}$ from a combination of SeCr, patient age and body size (Cockcroft and Gault 1976). In some transplant units this calculation is utilised in preference to SeCr to monitor patients at follow up. This formula was applied to both the first and second recorded measurements of SeCr which were separated by an interval of 3 months. On both occasions a good correlation existed between GFR and the Gault and Cockcroft modification of SeCr (Figs 4.2.vi and 4.2.vii). However, in patient follow-up, it is not single values but changes in SeCr which are used to represent and therefore monitor changes in renal function. To investigate this, changes measured in true GFR were plotted against changes calculated from the Gault & Cockcroft manipulation of SeCr (Fig 4.2.viii). Despite a positive, significant correlation between these two variables, the slope of the line of best-fit falls far from unity. Several patients appear in the area where true GFR has shown an improvement despite a rise in SeCr concentration. More worrying is the larger number of patients who demonstrated an improvement or fall in SeCr concentration but which was accompanied by a concomitant deterioration or fall in true GFR. No correlation was demonstrated between the amount by which Gault & Cockcroft incorrectly calculated "GFR" and the true level of GFR ($p = 0.682$).
Correlation = 0.721
p < 0.001
slope = 0.99

FIG. 4.2. True GFR v Gault and Cockcroft calculation of "GFR".
FIG. 4.2.vi
"GFR" 3 months after [4.2.vi].

"GFR" v Gault and Cockcroft

Correlation = 0.786
p < 0.001
slope = 0.98

2nd Gault & Cockcroft "GFR" (ml/min)

2nd Isotope GFR (ml/min)

0 20 40 60 80 100 120

0 20 40 60 80 100
Change in Gault & Cockcroft "GFR" (ml/min)

Isotope GFR deteriorated in these patients yet SeCr improved

Correlation = 0.446
p < 0.002
slope + 0.39

Isotope GFR improved in these patients yet SeCr deteriorated

FIG. 4.2.viii Change in isotope GFR v change in Gault and Cockcroft "GFR".
CONCLUSION

From these results it is clear that SeCr concentrations used alone do not provide an accurate or reproducible indication of level of function in a renal transplant recipient. After manipulation, however, a single value of SeCr can provide an estimate of function and is also useful in the detection of acute renal allograft dysfunction. When monitoring renal allograft function, it must be borne in mind that a kidney with better function and a GFR in excess of 45ml/min, can suffer a marked loss of filtration capacity without demonstrating a comparable change in SeCr concentration. Consequently, when renal function deteriorates as a result of a CyA-induced chronic nephrotoxicity, relatively large functional losses will not become apparent until late in the progression of the condition.

Therefore, to identify and manage chronic CyA nephrotoxicity, renal transplant recipients should be regularly investigated by isotope measurements of GFR. Throughout the rest of this work, all assessments of renal functional included isotope measurements of GFR and ERPF.
4.3 THE EFFECT OF THE THROMBOXANE RECEPTOR ANTAGONIST, GR32191B, ON RENAL TRANSPLANT FUNCTION

INTRODUCTION

This study was performed to investigate the effect of a specific thromboxane receptor antagonist (GR32191B: Glaxo Group Research) on CyA nephrotoxicity and hence renal function in a group of renal transplant recipients who had received their transplant no longer than 2 years previously. The drug was given on a randomised, double-blind, placebo-controlled basis for a period of 3 months.

RESULTS

Numbers

A total number of 49 patients consented to participate in the GR32191B trial. One of these patients never commenced trial medication, having suffered an acute rejection episode during the week after agreeing to take part. This was followed by graft failure which subsequently required a transplant nephrectomy. 48 patients therefore commenced trial medication; there were 15 female and 33 male participants.

46 patients had received a first transplant, all but one being from a cadaver donor. One patient had a second and one a third transplant.

Medication

After breaking the code, it was discovered that 25 patients had received drug whilst 23 were prescribed placebo medication.

4.3.1 Major Adverse Event necessitating withdrawal from the trial

In total, 4 patients stopped their medication prior to the end of the trial. All were taking active drug and therefore only 21 of the 25 patients in the drug group completed the treatment period.
Patient number 10 stopped medication at week 7. This man had developed epigastric, indigestion-type pain over the previous 2 weeks, which was temporally related to the time of tablet ingestion. An upper gastrointestinal endoscopy was performed which demonstrated multiple, tiny ulcers at the lower end of the oesophagus. Medication with ranitidine was commenced and the pain settled rapidly. The patient was completely well 2 months after withdrawing from the trial. However, 3 months after this and 5 months after leaving the trial, this patient developed a different type of upper abdominal discomfort. Hepatomegaly was discovered on examination and an ultra-sound guided biopsy revealed a primary hepatocellular adenocarcinoma. The condition of this patient deteriorated rapidly and he died 7 months after stopping GR32191B.

Patient number 38 developed an episode of acute renal dysfunction 7 weeks after starting trial medication and 5 months post-transplantation. He was admitted for a renal transplant biopsy which was performed under local anaesthetic with ultra-sound guidance. No histological abnormality was demonstrated. Unfortunately during the procedure an intra-renal vessel had unknowingly been damaged and the patient required re-admission several hours after discharge because of gross haematuria. Surgical exploration was undertaken, the kidney requiring sutures and Sterispon to arrest bleeding. Trial medication was ceased by the surgeons in charge at this time.

Patient number 43 had suffered with epigastric pain prior to admission to the study. A diagnosis of Cytomegalovirus (CMV) duodenitis had been made 6 months previously. He had commenced on ranitidine at that time with some but not complete relief. He occasionally also took antacids to which the discomfort responded well. After 8 days of trial medication, he developed more severe epigastric pain which did not
respond to his usual antacids. An upper gastrointestinal endoscopy was performed which revealed duodenal inflammation with red, raised areas scattered over the mucosa. Biopsies of these demonstrated "a polyclonal lymphoproliferative lesion" thought to be associated with immunosuppression. Virology studies demonstrated an acute CMV infection within the duodenal mucosa which was thought to account for the lymphoid infiltrate. All immunosuppression was stopped and the patient was also treated with Ganciclovir (1,3-dihydroxy-2-propoxymethyl guanine). Despite the absence of immunosuppression for 4 weeks his transplanted kidney continued to function well with a marked improvement in serum creatinine levels. Once endoscopy appearances had returned to normal, despite the histological persistence of an infiltrate, immunosuppression with azathioprine was commenced. Six months after the original biopsy diagnosis, no histological abnormalities could be detected within duodenal tissue and the patient continues in good health to the present time.

Patient Number 44 ceased taking trial medication after only 10 days. She complained of headaches and nausea closely related to the time of tablet ingestion. She was also perturbed by the number of tablets required each day. This was compounded by her prednisolone dose of 30mg on alternate days, which was prescribed as six x 5mg tablets. Her symptoms resolved completely within 2 days of stopping the trial drug.

4.3.ii Malignancy and Mortality

The events in this study indicate a 4% incidence of malignancy (1/25) in patients receiving active drug compared with 0 in the placebo group. This value falls within described ranges for patients receiving immunosuppressive therapy (Hanto and Simmons 1986). However, as no patients receiving placebo developed a malignancy, a carcinogenic effect
cannot be excluded.

One patient in this study developed severe bleeding more than 24 hours after a fine-needle transplant biopsy. Despite this coming from a large vessel requiring sutures to arrest haemorrhage, the late presentation may indicate inadequate clot formation to arrest bleeding once the patient had mobilised and returned home. All patients routinely remain on 24-hour bed rest with regular observation of vital signs after a transplant biopsy. Therefore, GR32191B may be incriminated in these circumstances.

4.3.iii Minor Adverse Events

The trial was conducted from January 1988 until February 1989. During the first 3 months, many patients were recruited and participated. The numbers then decreased rapidly, new recruits being provided only from recent renal transplant recipients. Consequently, there was a high incidence of upper respiratory tract infection (URTI) and influenza in both groups of patients, who participated in the winter months. 17 patients reported symptoms overall, 7 taking active drug and 12 placebo. No significant difference was noted, however, between the two groups.

Nausea and indigestion are common symptoms in renal transplant recipients, especially in those receiving steroids as part of their immunosuppressive regimen. During the treatment period, patients in both groups reported these symptoms but with no significant difference between drug or placebo groups. Diarrhoea and vomiting (D & V) could in all cases be related to a foodstuff ingested and was not thought to relate to trial medication:
<table>
<thead>
<tr>
<th>Drug</th>
<th>DRUG</th>
<th>PLACEBO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indigestion</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Nausea</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>D &amp; V</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

Ankle oedema occurs relatively often in patients with renal dysfunction and is also a recognised side effect of the calcium-channel antagonist nifedipine, which all patients were receiving:

Ankle oedema 2 6

Headache was reported as a side effect of GR32191B in studies of normal human volunteers. 3 patients taking the active drug reported developing headaches. In two patients these were mild and transient, not persisting throughout the 3-month study period. In one patient this symptom necessitated drug withdrawal. No patients from the placebo group reported headache.

One patient developed a urinary tract infection on GR32191B and one a purpuric skin rash which persisted beyond treatment. Two patients acquired Herpes Zoster infection during the medication period, however, both were taking placebo.

4.3.iv Transplant variables and baseline function

Using the Mann Whitney-U test, baseline data from the placebo- and drug-treatment groups were examined and compared. Except for a greater number of total mismatches detected in the placebo group, no other significant differences were noted between the two patient groups in any other variable relating to transplantation or immunology (Table 4.3.i).

Cyclosporin dosages and trough whole-blood levels also compared well at the onset of the trial.
<table>
<thead>
<tr>
<th></th>
<th>DRUG</th>
<th>PLACEBO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>25</td>
<td>23</td>
</tr>
<tr>
<td>Female : Male</td>
<td>8:17</td>
<td>7:16</td>
</tr>
<tr>
<td>Patient age</td>
<td>44.8 (15.9)</td>
<td>42.0 (12.8)</td>
</tr>
<tr>
<td>Donor age</td>
<td>37.6 (13.9)</td>
<td>32.5 (17.9)</td>
</tr>
<tr>
<td>Time since transplant</td>
<td>11.9 (8.0)</td>
<td>11.7 (8.5)</td>
</tr>
<tr>
<td>Cold time</td>
<td>17.35 (17.1)</td>
<td>18.37 (6.6)</td>
</tr>
<tr>
<td>1st warm time</td>
<td>2.84 (2.9)</td>
<td>2.7 (2.8)</td>
</tr>
<tr>
<td>2nd warm time (anastomosis)</td>
<td>30.8 (6.8)</td>
<td>31.1 (6.9)</td>
</tr>
<tr>
<td>Rejections</td>
<td>0.24 (6/16pts)</td>
<td>0.7 (16/10pts)</td>
</tr>
<tr>
<td>Acute CyA toxicity</td>
<td>0.16 (4/4pts)</td>
<td>0.04 (1/1pt)</td>
</tr>
<tr>
<td>Ureteric obstructions</td>
<td>0</td>
<td>0.13 (3/3pts)</td>
</tr>
<tr>
<td>Severe infections</td>
<td>0.16 (4/4pts)</td>
<td>0.26 (6/5pts)</td>
</tr>
<tr>
<td>Diabetics</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Mismatch A</td>
<td>1 (0.58)</td>
<td>0.91 (0.51)</td>
</tr>
<tr>
<td>B</td>
<td>0.84 (0.69)</td>
<td>1.13 (0.55)</td>
</tr>
<tr>
<td>D</td>
<td>1.12 (0.73)</td>
<td>1.22 (0.6)</td>
</tr>
<tr>
<td><strong>TOTAL MM</strong></td>
<td><strong>2.96 (0.93)</strong></td>
<td><strong>3.26 (0.86)</strong></td>
</tr>
<tr>
<td>ATN</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>CyA dose</td>
<td>6.1 (1.73)</td>
<td>6.67 (2.29)</td>
</tr>
<tr>
<td>CyA level</td>
<td>308 (105)</td>
<td>394 (248)</td>
</tr>
</tbody>
</table>

*Table 4.3.i* Recipient and transplant variables before commencing trial medication.
Measured baseline renal function was also similar in the randomly selected groups of patients. (mean values +/- 1SD)

<table>
<thead>
<tr>
<th></th>
<th>DRUG</th>
<th>PLACEBO</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFR</td>
<td>44.7 (12.03)</td>
<td>45.7 (12.7)</td>
</tr>
<tr>
<td>ERPF</td>
<td>226.7 (66.2)</td>
<td>242 (101)</td>
</tr>
<tr>
<td>SeCr</td>
<td>146.9 (43.78)</td>
<td>158.7 (64.2)</td>
</tr>
<tr>
<td>ClCr</td>
<td>56.1 (23.8)</td>
<td>62.4 (22.6)</td>
</tr>
<tr>
<td>BP(s)</td>
<td>136.3 (20.8)</td>
<td>133.5 (22.8)</td>
</tr>
<tr>
<td>BP(d)</td>
<td>84.2 (4.98)</td>
<td>82.6 (11.7)</td>
</tr>
<tr>
<td>Protein</td>
<td>0.31 (0.22)</td>
<td>0.41 (0.33)</td>
</tr>
</tbody>
</table>

SUBGROUPS

44 patients therefore completed the trial, 21 receiving drug and 23 placebo.

Patients were further subdivided into groups which depended upon the time-interval since transplantation:

<table>
<thead>
<tr>
<th>TIME SINCE TRANSPLANT</th>
<th>DRUG</th>
<th>PLACEBO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (6wks-6 months)</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>Group 2 (6-12 months)</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Group 3 (12-18 months)</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Group 4 (18-24 months)</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>TOTAL</td>
<td>21</td>
<td>23 PATIENTS</td>
</tr>
</tbody>
</table>

[As the numbers of patients in groups 2 and 3 were too small for statistical analysis, the results from these two groups have been added and analysed together throughout the study.]
Comparing Groups (Biochemistry, Haematology, Renal Function, CyA)

When baseline measurements were compared between patients in each timed group, some slight but significant differences were noted. (p = 0.05) (Mean values +/-1 SD)

**Group 1**

i) SeCr levels were lower in the drug group

118.3 (20.3) v 170.3 (66.1) \( \mu \text{mol/l} \)

ii) Total serum protein level was higher in the drug group

67.29 (8.58) v 60.89 (6.7) g/l

iii) Platelet count was higher in the drug group

413.7 (128) v 306.8 (76.9) \( \times 10^9 /l \)

**Group (2+3)**

i) Alanine transaminase was lower in the drug group

10.86 (3.89) v 33 (19.16) IU/l

**Group 4**

i) Platelet count was higher in the drug group

363.6 (115) v 269.4 (51.5) \( \times 10^9 /l \)

ii) Cyclosporin A dose was lower in the drug group

5.25 (1.13) v 6.79 (1.97) mg/kg

Throughout this study, a very large number of statistical analyses were performed. Most laboratory measurements analysed have a quoted coefficient of variation of at least 10%. Consequently, accepting statistical significance at the 5% level will include a proportion of "significant" results which are not true differences but which result from wide variations in the values measured. The majority of values quoted above fall within the normal range and are thought to result from these acceptable variations.
Rejections

Patients in Group 4 had suffered fewer early acute rejection episodes than those in Group (2+3) or Group 1. In the patients who subsequently received placebo, these differences attained statistical significance when Groups 1 and 4 were compared. This is an important factor which must be considered when examining differences in renal functional demonstrated between the individual Groups.

<table>
<thead>
<tr>
<th></th>
<th>DRUG</th>
<th>PLACEBO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>Group 2+3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Group 4</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>TOTAL</td>
<td>6</td>
<td>16</td>
</tr>
</tbody>
</table>

* Group 1 (placebo) patients overall had significantly more acute rejection episodes than Group 4  \( p = 0.05 \)

4.3.v  RENAL FUNCTION - After 3 months' treatment with PLACEBO

All patients (n=23)

There were no significant changes in renal function when the results from all patients receiving placebo were analysed. While mean GFR showed a slight tendency to improve, ERPF, \( \text{Cl}_{\text{CR}} \) and SeCr demonstrated marked stability over this short period of time.
<table>
<thead>
<tr>
<th></th>
<th>PRE-</th>
<th>POST-PLACEBO</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFR</td>
<td>45.71 (12.7)</td>
<td>47.7 (17.5) ml/min</td>
</tr>
<tr>
<td>ERPF</td>
<td>242.6 (101)</td>
<td>242.0 (103.9) ml/min</td>
</tr>
<tr>
<td>CIqj</td>
<td>62.4 (22.6)</td>
<td>62.8 (35.6) ml/min</td>
</tr>
<tr>
<td>SeCr</td>
<td>158.7 (64.2)</td>
<td>153.1 (63.8) μmol/l</td>
</tr>
</tbody>
</table>

Blood Pressure (BP) and Renal Vascular Resistance (RVR) also showed minimal change in this group of patients.

<table>
<thead>
<tr>
<th></th>
<th>PRE-</th>
<th>POST-PLACEBO</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP(s)</td>
<td>133.5 (22.8)</td>
<td>129.1 (16) mmHg</td>
</tr>
<tr>
<td>BP(d)</td>
<td>82.6 (11.7)</td>
<td>79.8 (9.1) mmHg</td>
</tr>
<tr>
<td>RVR</td>
<td>50.6 (26.4)</td>
<td>47.2 (20.4) dyne sec/cm²</td>
</tr>
</tbody>
</table>

No change in CyA doses or trough levels was demonstrated.

<table>
<thead>
<tr>
<th></th>
<th>PRE-</th>
<th>POST-PLACEBO</th>
</tr>
</thead>
<tbody>
<tr>
<td>CyA dose</td>
<td>6.67 (2.3)</td>
<td>6.26 (1.9) mg/kg</td>
</tr>
<tr>
<td>CyA level</td>
<td>394.1 (247.5)</td>
<td>344.0 (200.1) ng/ml</td>
</tr>
</tbody>
</table>

**Individual groups**

**Group 1 (n=9)**

As one would expect, there was a tendency for CyA dosage and associated whole-blood trough level to decrease during the first 6 months after transplantation. However, the decrements recorded did not reach statistical significance:
This group demonstrated deterioration in measurements of renal function over the early post-transplant period, although this was not reflected in the serum concentrations of creatinine which suggested improvement. Mean and median values differed greatly and therefore median values are quoted. Despite this deterioration, none of the changes achieved significance.

**median values**

<table>
<thead>
<tr>
<th></th>
<th>PRE-</th>
<th>POST-PLACEBO</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFR</td>
<td>43.8</td>
<td>34.5</td>
</tr>
<tr>
<td>ERPF</td>
<td>199.5</td>
<td>185.5</td>
</tr>
<tr>
<td>ClCr</td>
<td>53.4</td>
<td>40</td>
</tr>
<tr>
<td>SeCr</td>
<td>157</td>
<td>137</td>
</tr>
</tbody>
</table>

BP and RVR remained constant over the same period

<table>
<thead>
<tr>
<th></th>
<th>PRE-</th>
<th>POST-PLACEBO</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP(s)</td>
<td>131.7 (16.4)</td>
<td>129.4 (16.7)</td>
</tr>
<tr>
<td>BP(d)</td>
<td>82.8 (8.3)</td>
<td>78.9 (7.8)</td>
</tr>
<tr>
<td>RVR</td>
<td>52.6 (21)</td>
<td>51.9 (22.2)</td>
</tr>
</tbody>
</table>

**Group (2+3) (n=6)**

This represents a collection of more stable patients in whom the CyA dose-reducing regimen had been completed. However, during the previous months of CyA therapy, periods of relatively high toxic dosing may have been experienced. Over the 3-month study period, SeCr alone
demonstrated a significant improvement whilst both ERPF and $Cl_{Cr}$ deteriorated but this did not reach significance.

<table>
<thead>
<tr>
<th>PRE-</th>
<th>POST-PLACEBO</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFR</td>
<td>44.8 (12.5)</td>
</tr>
<tr>
<td>ERPF</td>
<td>242 (9109)</td>
</tr>
<tr>
<td>$Cl_{Cr}$</td>
<td>62.9 (33.4)</td>
</tr>
<tr>
<td>SeCr</td>
<td>185.5 (66,2)</td>
</tr>
<tr>
<td>BP(s)</td>
<td>134.2 (31.7)</td>
</tr>
<tr>
<td>BP(d)</td>
<td>78 (11.6)</td>
</tr>
<tr>
<td>RVR</td>
<td>50.5 (32.3)</td>
</tr>
</tbody>
</table>

* $p = 0.05$

Group 4  (n=8)

This group of patients demonstrated remarkably constant function over the short study period. This group also demonstrated better renal function than any of the other groups despite comparable doses of CyA.

<table>
<thead>
<tr>
<th>PRE-</th>
<th>POST-PLACEBO</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFR</td>
<td>51.3 (12.1)</td>
</tr>
<tr>
<td>ERPF</td>
<td>269.9 (113.6)</td>
</tr>
<tr>
<td>$Cl_{Cr}$</td>
<td>70.6 (11.7)</td>
</tr>
<tr>
<td>SeCr</td>
<td>125.4 (52.5)</td>
</tr>
<tr>
<td>CyA dose</td>
<td>6.79</td>
</tr>
</tbody>
</table>

Group 1 contains patients in whom stability of function is yet to be achieved. CyA doses are being reduced and at the same time the risks of acute cellular rejection, infection and episodes of acute CyA
nephrotoxicity are greater. Results from Group 2+3+4 can be added to produce a larger stable population more suitable for statistical analysis.

**Group 2+3+4 (n=14)**

In this larger group, no significant changes were demonstrated in any parameter over the period of treatment with placebo.

4.3.vi RENAL FUNCTION - After 3 months' treatment with GR32191B

**All patients (n=21)**

When the results from all patients treated with GR32191B were analysed a significant decrease in GFR was recorded. The other measured indicators of renal function showed small but non-significant deteriorations.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pre-GR32191B</th>
<th>Post-GR32191B</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFR</td>
<td>46.0 (12.1)</td>
<td>40.2 (13.9)</td>
<td>ml/min *</td>
</tr>
<tr>
<td>ERPF</td>
<td>238.4 (63.2)</td>
<td>219.8 (69.9)</td>
<td>ml/min</td>
</tr>
<tr>
<td>ClCr</td>
<td>57.1 (24.7)</td>
<td>56.9 (25.2)</td>
<td>ml/min</td>
</tr>
<tr>
<td>SeCr</td>
<td>146.9 (45.9)</td>
<td>155.8 (49.8)</td>
<td>μmol/l</td>
</tr>
</tbody>
</table>

\*p = 0.025

In the treated patients, BP and RVR showed no trends, remaining constant throughout the study. A small change in CyA dosage from 6.11 (SD 1.85) to 5.98 (SD 1.59) mg/kg overall, was also not significant (Appendix VI).

**Individual groups**

**Group 1 (n=7)**

As in the comparable placebo group, CyA dosages decreased during the
study period, however, the mean dose both before and after treatment was higher. In addition mean and median CyA trough levels measured showed a non-significant increase.

<table>
<thead>
<tr>
<th></th>
<th>PRE-</th>
<th>POST-GR3219B</th>
</tr>
</thead>
<tbody>
<tr>
<td>CyA dose</td>
<td>7.4 (range 4.5-10)</td>
<td>6.64 (range 4-9) mg/kg</td>
</tr>
<tr>
<td>mean CyA level</td>
<td>325.7 (153)</td>
<td>345.7 (151.3) ng/ml</td>
</tr>
<tr>
<td>median CyA level</td>
<td>275</td>
<td>333          ng/ml</td>
</tr>
</tbody>
</table>

Significant deteriorations in both ERPF and SeCr occurred over the treatment period. This rendered the 3-month SeCr levels similar in both drug and placebo groups, as at the onset of the trial, the former had a very much lower SeCr level.

The rise in SeCr cannot be attributed to excessive loss of function from rejection during the trial period as only two Group 1 patients suffered an acute rejection episode during the trial, one on active drug and one taking placebo. An equal and moderate loss of function occurred in both patients, SeCr levels being elevated to 20μmol/l above their pre-rejection values.

<table>
<thead>
<tr>
<th></th>
<th>PRE-</th>
<th>POST-GR3219B</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFR</td>
<td>46.7 (11.9)</td>
<td>37.9 (9.1)   ml/min</td>
</tr>
<tr>
<td>ERPF</td>
<td>270.7 (78.4)</td>
<td>222.5 (67.5) ml/min *</td>
</tr>
<tr>
<td>ClCr</td>
<td>61.1 (26.9)</td>
<td>50.0 (12.1)  ml/min</td>
</tr>
<tr>
<td>SeCr</td>
<td>118.3 (19)</td>
<td>145.3 (32.7) μmol/l *</td>
</tr>
</tbody>
</table>

* p = 0.05

Despite the significant decrease in ERPF, BP and RVR remained stable in this group during treatment with GR32191B (Appendix VI).
Group (2+3)  \((n=6)\)

No significant changes were noted in any parameter measured in this group. Again a deterioration in GFR was demonstrated over the short treatment period.

<table>
<thead>
<tr>
<th></th>
<th>PRE-</th>
<th>POST-GR32191B</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>GFR</td>
<td>47.3 (15.2)</td>
<td>43.6 (17.6)</td>
<td>ml/min</td>
</tr>
<tr>
<td>ERPF</td>
<td>223 (45.5)</td>
<td>227 (74.9)</td>
<td>ml/min</td>
</tr>
<tr>
<td>ClCr</td>
<td>58.3 (32.9)</td>
<td>52.6 (17.1)</td>
<td>ml/min</td>
</tr>
<tr>
<td>SeCr</td>
<td>183 (55.1)</td>
<td>176.5 (62)</td>
<td>pmol/l</td>
</tr>
</tbody>
</table>

These patients demonstrated a tendency for both systolic and diastolic BP to increase during the treatment period and despite no change in ERPF this led to a marked increase in RVR.

<table>
<thead>
<tr>
<th></th>
<th>PRE-</th>
<th>POST-GR32191B</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>BP(s)</td>
<td>140 (20.9)</td>
<td>148.3 (19.4)</td>
<td>mmHg</td>
</tr>
<tr>
<td>BP(d)</td>
<td>83.3 (17.5)</td>
<td>90 (8.9)</td>
<td>mmHg</td>
</tr>
<tr>
<td>RVR</td>
<td>47.5 (13.9)</td>
<td>55.52 (27.3)</td>
<td>dyne sec/cm$^2$</td>
</tr>
</tbody>
</table>

There were no changes in CyA dosage or trough levels (Appendix VI).

Group 4  \((n=8)\)

No significant changes were recorded in any measured variable, however, again the trend was for renal function to deteriorate.
Neither systolic nor diastolic BP showed any tendency to deteriorate, whilst RVR increased only slightly (Appendix VI).

**Group 2+3+4 (n=14)**

When these Groups were combined, only the deterioration demonstrated in GFR achieved statistical significance. The small changes in other parameters measured tended to cancel each other, demonstrating no final overall significant changes.

<table>
<thead>
<tr>
<th></th>
<th>PRE-</th>
<th>POST-GR32191B</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFR</td>
<td>45.4 (11.4)</td>
<td>39.6 (15.5)</td>
</tr>
<tr>
<td>ERPF</td>
<td>221.6 (56.5)</td>
<td>212.1 (77.1)</td>
</tr>
<tr>
<td>ClCr</td>
<td>56.1 (20.1)</td>
<td>67.7 (37.6)</td>
</tr>
<tr>
<td>SeCr</td>
<td>145 (40)</td>
<td>149.4 (57.3)</td>
</tr>
<tr>
<td>RVR</td>
<td>49 (14.6)</td>
<td>54.5 (23.2)</td>
</tr>
</tbody>
</table>

*P = 0.041

CyA dose and trough levels remained unchanged (Appendix VI).
4.3.vii Biochemistry, Haematology and Urinalysis

After 3 months' treatment with PLACEBO (significant changes)

Group 1 (n=9)

- Over the 3-month treatment period a small but significant fall in alanine transaminase was noted from 28 (SD 15.8) to 16.9 (SD 14.3) IU/1.

- Serum protein levels increased from 60.9 (SD 6.7) to 66.1 (SD 7.5) g/l, bringing the serum protein level to a concentration which is comparable with that of the drug-treated group prior to the onset of the study.

- MCV also decreased over the study period from 85.8 (SD 5.6) to 79.6 (SD 6.5) fl.

- 24-hour protein excretion decreased from 0.54 (SD 0.34) to 0.23 (SD 0.09) g/24hr

Group 2+3 (n=6)

- A decrease in serum calcium occurred from 2.53 (SD 0.21) to 2.38 (SD 0.1) mmol/l

Group 4 (n=8)

- A decrease in MCV was recorded from 86.6 (SD 6.5) to 85.3 (SD 6.3) fl.

All patients (n=23)

Overall significant changes in serum and urinary protein, ALT and MCV occurred during the study period in those taking placebo.
<table>
<thead>
<tr>
<th></th>
<th>PRE-</th>
<th>POST-PLACEBO</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>24.9 (16.4)</td>
<td>18 (11.5)</td>
<td>IU/1</td>
</tr>
<tr>
<td>MCV</td>
<td>86.6 (5.4)</td>
<td>83.1 (7.2)</td>
<td>fl.</td>
</tr>
<tr>
<td>Serum protein</td>
<td>66.0 (7.3)</td>
<td>69.1 (7.2)</td>
<td>g/l</td>
</tr>
<tr>
<td>Urinary protein</td>
<td>0.41 (.33)</td>
<td>0.28 (.27)</td>
<td>g/24hr</td>
</tr>
</tbody>
</table>

After 3 months' treatment with GR32191B (significant changes)

Group 1 (n=7)
- This group also demonstrated a decrease in MCV over the study period from 85.5 (SD 7.9) to 80.1 (SD 7.5)fl.

Group 2+3 (n=6)
- No significant changes occurred.

Group 4 (n=8)
- An increase in serum glucose concentration was recorded but remained within the normal range changing from 4.9 (SD 0.6) to 5.4 (SD 0.5) mmol/1.
- ALT also decreased over the treatment period from 20.9 (SD 10.2) to 14.4 (SD 5.1) IU/1.

All patients (n=21)
Overall significant changes in serum bilirubin and calcium were noted in addition to a decrease in MCV.
Pre-GR32191B v post-placebo

No significant differences were demonstrated in any measurement when compared in this way.

4.3.viii Prostanoid Concentrations

1. Urinary Prostanoid Metabolites

A total of 42 patients had urinary prostanoid metabolite concentrations measured by radioimmunoassay; 19 of these completed treatment with GR32191B and 21 with the placebo. 32 measurements of the same urinary metabolites were also performed 3 months after ceasing either active drug or placebo. These later values were used to examine longer term trends in the ratio of prostanoids excreted.

No significant differences were demonstrated in the concentrations of either TXB$_2$ or 6kPGF$_{1a}$ between individual groups or the overall patient population prior to treatment (Appendix VII).

<table>
<thead>
<tr>
<th></th>
<th>Pre-Treatment [ng/24hour]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Drug Group (21)</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>TXB$_2$</td>
<td>1998.0 (1030.9)</td>
</tr>
<tr>
<td>6kPGF$_{1a}$</td>
<td>1332.5 (455.3)</td>
</tr>
</tbody>
</table>
Thromboxane B₂

After PLACEBO

After 3 months on placebo, a significant increase in excretion of the urinary metabolite TXB₂ was demonstrated. The changes measured in the individual Groups 1 and 4 also reached significance.

TXB₂ (n=21) [ng/24hr]

<table>
<thead>
<tr>
<th>Group</th>
<th>PRE-</th>
<th>POST-PLACEBO</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1704.3 (796.1)</td>
<td>3207.8 (2164.5)</td>
<td>0.05</td>
</tr>
<tr>
<td>2+3</td>
<td>2049.5 (823.4)</td>
<td>3419.2 (1580)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1995.6 (853.3)</td>
<td>2925.4 (1570.4)</td>
<td>0.05</td>
</tr>
<tr>
<td>ALL</td>
<td>1900 (796.8)</td>
<td>3174.1 (1744)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

After GR32191B

3 months' treatment with the thromboxane A₂ receptor antagonist GR32191B, also produced a significant increase in the excretion of TXB₂. The increases demonstrated by individual Groups did not, however, achieve significance.

TXB₂ (n=19) [ng/24hr]

<table>
<thead>
<tr>
<th>Group</th>
<th>PRE-</th>
<th>POST-GR32191B</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2265.7 (1115.7)</td>
<td>3174.2 (1988)</td>
<td></td>
</tr>
<tr>
<td>2+3</td>
<td>1236.8 (516)</td>
<td>2597.2 (1419.9)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2094.4 (860.8)</td>
<td>2441.6 (1025.4)</td>
<td></td>
</tr>
<tr>
<td>ALL</td>
<td>1922.8 (936.0)</td>
<td>2713.9 (1436)</td>
<td>0.049</td>
</tr>
</tbody>
</table>
6-keto prostaglandin F\textsubscript{1} alpha

After PLACEBO

After 3 months' treatment with placebo, no significant changes in the urinary excretion of this substance were recorded in individual Groups nor when the results were combined.

\[
\begin{array}{c c c c c}
6kPGF\textsubscript{1a} (n=21) & [\text{ng/24hr}] \\
\hline
\text{PRE-} & \text{POST-PLACEBO} \\
6kPGF\textsubscript{1a} & 1297.5 (582.7) & 1128.5 (425.7) \\
\end{array}
\]

After GR32191B

After 3 months' treatment with the thromboxane receptor antagonist GR32191B, again, no overall significant changes occurred. There was also, however, a similar trend for 6kPGF\textsubscript{1a} levels to decrease and this reached a significant change only in Group 1.

\[
\begin{array}{c c c c c}
6kPGF\textsubscript{1a} (n=19) & [\text{ng/24hr}] \\
\hline
\text{PRE-} & \text{POST-GR32191B} \\
\text{Group 1} & 1739.3 (525) & 1051.8 (436.5) & p = 0.05 \\
\text{ALL} & 1372.5 (460.5) & 1095.4 (408.4) \\
\end{array}
\]

The ratio : 6kPGF\textsubscript{1a} /TXB\textsubscript{2} in urine

Another way of examining the relationship between these counteractive prostanoids is to calculate the ratio of metabolites i.e. 6kPGF\textsubscript{1a}/TXB\textsubscript{2}. Changes in this ratio will indicate if the overall response to a particular stimulus or therapy, is one of intra-renal vasoconstriction or vasodilatation.
A significant decrease in this ratio, consistent with increased total vasoconstrictory activity, was recorded following treatment with both placebo and GR32191B. However, as no differences were demonstrated between the two treatment groups, no overall drug effect was assumed.

When these urinary prostanoid ratios were calculated 3 months after ceasing all trial therapy, again a similar but non-significant increase was recorded in both drug and placebo groups. It is most likely that these results are demonstrating naturally occurring wide variations in urinary prostanoid metabolite concentration, which are unaffected by GR32191B.

<table>
<thead>
<tr>
<th>6kPGF$_1$a /TXB$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td><strong>PRE-</strong></td>
</tr>
<tr>
<td>Placebo 0.71 (0.26)</td>
</tr>
<tr>
<td>GR32191B 0.8 (0.41)</td>
</tr>
</tbody>
</table>

* p = 0.003  
** p = 0.002  

2. PLASMA PROSTANOID METABOLITES

At the onset of the study, it was believed that urinary prostanoid metabolites closely reflected intra-renal prostaglandin synthesis. It was felt that if GR32191B affected the production of these compounds, this would be demonstrated by these measurements. Therefore, for comparison only, the plasma from a small sample of patients, (the first 5 participants from each timed Group), was subjected to the same RIA's. One plasma sample was found to be unsuitable for assay and therefore, 19 samples were tested, 7 from patients who received GR32191B and 12 from those who received placebo. Analysis was confined to these two groups.
with no subdivision. All plasma prostanoid values fell within the normally accepted ranges described by other workers (Ritter et al 1983, Catella et al 1986) (Appendix VIII).

**Thromboxane B<sub>2</sub>**

**After GR32191B and PLACEBO**

No significant changes in the plasma concentrations of this substance were noted after treatment with either a thromboxane A<sub>2</sub> receptor antagonist (GR32191B) or placebo.

<table>
<thead>
<tr>
<th>PLACEBO</th>
<th>TXB&lt;sub&gt;2&lt;/sub&gt;</th>
<th>(n=12)</th>
<th>[ng/l = pg/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEAN</td>
<td>1SD</td>
<td>RANGE</td>
</tr>
<tr>
<td>PRE-</td>
<td>137.75</td>
<td>104.44</td>
<td>22-396</td>
</tr>
<tr>
<td>POST-</td>
<td>133.33</td>
<td>74.12</td>
<td>41-277</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GR32191B</th>
<th>TXB&lt;sub&gt;2&lt;/sub&gt;</th>
<th>(n=7)</th>
<th>[ng/l = pg/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEAN</td>
<td>1SD</td>
<td>RANGE</td>
</tr>
<tr>
<td>PRE-</td>
<td>100.57</td>
<td>35.55</td>
<td>55-157</td>
</tr>
<tr>
<td>POST-</td>
<td>144</td>
<td>103.54</td>
<td>81-373</td>
</tr>
</tbody>
</table>

**6-keto prostaglandin F<sub>1 alpha</sub>**

**After PLACEBO**

After 3 months' treatment with placebo, no change in the serum concentration of this metabolite was noted.
**PLACEBO**  

<table>
<thead>
<tr>
<th>6kPGF$_{1a}$ (n=12)</th>
<th>[ng/l = pg/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MEAN</strong></td>
<td><strong>1SD</strong></td>
</tr>
<tr>
<td><strong>PRE</strong>-</td>
<td>2.51</td>
</tr>
<tr>
<td><strong>POST</strong>-</td>
<td>2.58</td>
</tr>
</tbody>
</table>

After **GR32191B**

However, after 3 months' treatment with the thromboxane A$_2$ receptor antagonist, GR32191B, a significant decrease in the plasma concentration of 6kPGF$_{1a}$ was recorded, the mean concentration being reduced by 30%.

**GR32191B**  

<table>
<thead>
<tr>
<th>6kPGF$_{1a}$ (n=7)</th>
<th>[ng/l = pg/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MEAN</strong></td>
<td><strong>1SD</strong></td>
</tr>
<tr>
<td><strong>PRE</strong>-</td>
<td>2.59</td>
</tr>
<tr>
<td><strong>POST</strong>-</td>
<td>1.82</td>
</tr>
</tbody>
</table>

** p = 0.05 difference pre- and post-GR32191B  

* p = 0.05 difference between placebo and drug groups after therapy.

The ratio : 6kPGF$_{1a}$/TXB$_2$ in plasma

This ratio was again calculated to demonstrate the overall prostanoid activity within the plasma which resulted from treatment with GR32191B. A significant decrease in this ratio was demonstrated, only in patients receiving active drug. This suggests that the TXA$_2$ receptor antagonist, GR32191B, shifted the balance of these circulating prostanoids in favour of increased vasoconstriction.

<table>
<thead>
<tr>
<th>6kPGF$_{1a}$/TXB$_2$</th>
<th><strong>PRE</strong>-</th>
<th><strong>POST</strong>-TREATMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>GR32191B</td>
<td>0.03</td>
<td>0.02 * p = 0.05</td>
</tr>
</tbody>
</table>

205
4.3.ix Urinary Biochemistry

Aliquots from the 24-hour urinary collections were also assayed to investigate tubular function, before and after treatment with the thromboxane receptor antagonist. These measurements were made in 14 patients receiving active drug and in 20 receiving placebo.

The following measurements were made (Appendix IX):-

1. Glucose excretion (expressed as mmol/mmol of creatinine)
2. Fractional excretion of Sodium. Calculated from:
   \[
   \frac{\text{UNa/PNa}}{\text{UCr/PCr}} \times 100 \%
   \]
3. Sodium excretion (mmol/l)
4. Potassium excretion (mmol/l)
5. N-acetyl-d-glucosaminidase excretion (NAG) (IU/mmol Cr)

PLACEBO

Combining the 20 results, fractional excretion of sodium decreased significantly over the study period from a mean value of 1.74% (SD 1.07) to 1.27% (SD 0.86), \( p = 0.025 \). No other significant changes were noted in any other parameter measured whilst taking placebo.

GR32191B

Patients receiving active drug, demonstrated no significant changes in any parameter measured after the treatment period.

CONCLUSION

In this study, 3 patients developed headaches; in one case the severity necessitated withdrawal from the trial. Headache is a recognised side effect of this drug. Apart from this, no other minor side effects could
be attributed to GR32191B.

Treatment with GR32191B appeared to be associated with a significant deterioration in renal function, measured as GFR, when the combined data from all patients treated with active drug were analysed.

This deterioration occurred in parallel with a significant decrease in the circulating concentrations of the prostacyclin metabolite, 6-keto prostaglandin $F_1\alpha$.

Plasma concentrations of the prostanoid metabolites $\text{TXB}_2$ and $6\text{kPGF}_{1\alpha}$, measured both before and after treatment, fell within the normal ranges quoted by other workers using similar methods of collection and quantitation regardless of whether drug or placebo was taken (Appendix VIII).

Urinary concentrations of $6\text{kPGF}_{1\alpha}$ remained within the normal ranges quoted for healthy volunteers (Appendix VII). As anticipated, from studies of humans and animals receiving CyA therapy, urinary concentrations of $\text{TXB}_2$ were elevated above the quoted normal ranges.
4.4 THE ACUTE EFFECT OF CYCLOSPORIN A UPON RENAL FUNCTION

INTRODUCTION
This study was undertaken to demonstrate any acute effect upon renal haemodynamics that might follow a single oral dose of CyA. Firstly, 4 renal allograft recipients were investigated, taking their normal morning dose of CyA. Following this 4 dermatology patients were studied. These people were planning to receive CyA treatment for severe psoriasis or acute atopic eczema and were given a much larger loading dose of CyA. It was hoped that the latter group of patients might provide a suitable model in which the acute haemodynamic effects of CyA could be demonstrated, following which modification with GR32191B could be attempted.

RESULTS

4.4.1 Renal allograft patients
4 renal allograft recipients were investigated at 6, 8, and two at 22 months after transplantation. At the time of the study, they were taking between 4 and 5.6mg/kg of CyA per day in a divided dose. All 4 patients were also receiving ranitidine and low dose steroid therapy; none was taking a calcium-channel antagonist, whilst one was on atenolol and prazosin for the treatment of hypertension (RC).

Patient variables and baseline function are shown in Table 4.4.1
<table>
<thead>
<tr>
<th></th>
<th>JS</th>
<th>SMC</th>
<th>RC</th>
<th>HW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time since transplant</td>
<td>6</td>
<td>8</td>
<td>22</td>
<td>22 months</td>
</tr>
<tr>
<td>Age (sex)</td>
<td>28 (F)</td>
<td>34 (F)</td>
<td>47 (M)</td>
<td>22 (F) years</td>
</tr>
<tr>
<td>CyA dose</td>
<td>4.0</td>
<td>5.6</td>
<td>5.2</td>
<td>4.7 mg/kg/d</td>
</tr>
<tr>
<td>CyA Trough level</td>
<td>123</td>
<td>146</td>
<td>129</td>
<td>177 ng/ml</td>
</tr>
<tr>
<td>SeCr</td>
<td>81</td>
<td>148</td>
<td>147</td>
<td>87 µmol/L</td>
</tr>
<tr>
<td>ClCr</td>
<td>62.3</td>
<td>54.1</td>
<td>56.8</td>
<td>47.5 ml/min</td>
</tr>
<tr>
<td>GFR</td>
<td>50.7</td>
<td>39.8</td>
<td>38.3</td>
<td>58.9 ml/min</td>
</tr>
<tr>
<td>ERPF</td>
<td>254.3</td>
<td>185.2</td>
<td>141.2</td>
<td>287.7 ml/min</td>
</tr>
<tr>
<td>FF</td>
<td>19.9</td>
<td>21.5</td>
<td>27.1</td>
<td>20.5 %</td>
</tr>
<tr>
<td>BP</td>
<td>100/80</td>
<td>130/100</td>
<td>140/90</td>
<td>110/70 mmHg</td>
</tr>
<tr>
<td>RVR</td>
<td>27.7</td>
<td>73.9</td>
<td>78.9</td>
<td>29.65 dyne/sec/cm²</td>
</tr>
</tbody>
</table>

*Table 4.4.1* Baseline function and variables in the transplant patients investigated.
Individual doses were small, each patient taking his/her normal morning
dose of CyA which was half of that recorded in Table 4.4.i. All doses
were within the manufacturers' recommended range for maintenance therapy
and also maintained whole-blood trough levels, measured by HPLC, within
an acceptable therapeutic range. The effects of this dose were
therefore observed superimposed on a background of long-term chronic CyA
administration. As the group was too small for collective analysis,
individual responses were charted graphically (Figs 4.4.i and 4.4.ii).

Constant flow rates were more difficult to maintain in these patients,
despite standard fluid loading and replacement as described (Appendix
X). Flow rates in the transplant patients ranged between 4.0 and 8.3
ml/min.

GFR and ERPF were closely paralleled throughout the study in all
patients and a significant correlation was demonstrated between these
two variables throughout this period (r = 0.6, p = 0.009).

No consistent changes in GFR or ERPF were noted in response to the CyA
doses taken.

All 4 patients demonstrated an early relatively small peak in CyA whole-
blood concentration, which did not exceed 30% of the baseline trough
level. This response was seen in 3 patients, 1 hour and in the other, 2
hours after ingesting the CyA. Following these peaks, two of the
patients also demonstrated a second much higher CyA peak concentration,
occurring towards the end of the 5th hour of the study. In one patient
(HW) this achieved 88% and in the other 76% (SMc) of the baseline trough
concentration.
FIG. 4.4.i Percentage change in GFR, ERPF and CyA level following a therapeutic dose of CyA in renal allograft recipients [JS] and [SMc].
FIG. 4.4.ii Percentage change in GFR, ERPF and CyA level following a therapeutic dose of CyA in renal allograft recipients [RC] and [HW].
When the recorded changes in GFR and ERPF were correlated with the changes in whole-blood CyA concentration, 1 patient only demonstrated a relationship.

A significant negative correlation between % changes in both GFR and ERPF and the measured % change in CyA level was demonstrated by patient JS:

Spearman correlation of % change in GFR and CyA level

\[ r = -0.93 \]
\[ p = 0.019 \]

Spearman correlation of % change in ERPF and CyA level

\[ r = -0.98 \]
\[ p = 0.003. \]

It can be seen (Fig 4.4.i) that the early rise in CyA blood concentration was associated with a fall in GFR and ERPF. However, later increases in GFR and ERPF occurred in the presence of persistent elevations of CyA level.

With this very small sample size and the small changes recorded in each parameter, it would be unwise to attribute causality to the relationships demonstrated.

4.4.ii Dermatology patients

Each patient in this group received a large, single, oral dose of 12mg of CyA per kg of body weight during the study. Following the study, a therapeutic, maintenance dose of 5mg /kg was given in a divided daily dose. Regular assessments of renal function were performed subsequently.

All patients noted marked improvement in their psoriasis within 2 to 3 days of commencing medication, with generalised softening of their skin.
and a decrease in size of plaques. After 2 weeks of therapy, plaques and scaling had virtually disappeared.

No patients developed any adverse symptoms as a result of the single, large, loading dose of CyA.

Baseline function and patient variables are shown in table 4.4.ii.

<table>
<thead>
<tr>
<th>CMc</th>
<th>IE</th>
<th>GP</th>
<th>IA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (sex)</td>
<td>25 (F)</td>
<td>31 (M)</td>
<td>18 (M)</td>
</tr>
<tr>
<td>ScCr</td>
<td>66</td>
<td>66</td>
<td>82</td>
</tr>
<tr>
<td>ClCr</td>
<td>139</td>
<td>111.3</td>
<td>128.7</td>
</tr>
<tr>
<td>GFR</td>
<td>96.5</td>
<td>101.9</td>
<td>132.3</td>
</tr>
<tr>
<td>ERPF</td>
<td>401.4</td>
<td>472.9</td>
<td>532.7</td>
</tr>
<tr>
<td>FF</td>
<td>24.0</td>
<td>21.5</td>
<td>24.8</td>
</tr>
<tr>
<td>BP</td>
<td>120/60</td>
<td>140/85</td>
<td>100/65</td>
</tr>
<tr>
<td>RVR</td>
<td>16.2</td>
<td>20.85</td>
<td>15.7</td>
</tr>
</tbody>
</table>

Table 4.4.ii Baseline function and variables in the dermatology patients investigated.

Regular urine flow rates of between 8 and 15 ml/min were easily maintained by all patients throughout the study period (Appendix X).

All patients demonstrated a peak CyA blood level measured by whole-blood HPLC between 1.5 and 2.25 hours after the oral dose. The size of the peak varied from 720 to 1130 ng/ml. Over the period of the investigation after peak concentration, a gentle fall in the CyA level was demonstrated in all patients, reaching approximately 50% of the peak concentration by the 5th hour after ingestion (Fig 4.4.iii and 4.4.iv).

214
FIG. 4.4.iii Percentage change in GFR and ERPF and simultaneous CyA concentration after 12mg/kg. [Dermatology patients CMc & IE]
FIG. 4.4.iv Percentage change in GFR and ERPF and simultaneous CyA concentration after 12mg/kg. [Dermatology patients GP &IA]
GFR and ERPF values paralleled each other throughout the study in all patients. When the changes in GFR were correlated with the changes in ERPF a significant relationship existed between these two parameters ($r = 0.79$, $p < 0.001$).

Both GFR and ERPF remained remarkably constant in all patients after the dose of CyA. No significant changes in these parameters occurred, despite the brief but high blood concentrations of CyA. No correlation could be demonstrated between either changes in GFR or ERPF and the CyA blood level recorded at the same point in time.

Patient IE alone demonstrated a slight trend for BP to rise during the study period. This patient also had a relatively high resting BP and RVR when compared with the other patients and has since developed hypertension on maintenance CyA therapy, necessitating dose reduction.

**CONCLUSION**

This study was unable to demonstrate any consistent, acute effect of CyA upon renal haemodynamics. In a group of renal transplant patients, their usual twice daily dose produced only a gentle increase in whole-blood CyA concentration which was not associated with clear changes in either GFR or ERPF. When a much larger, single oral dose of CyA was given to patients with normal renal function, despite recording much higher whole-blood CyA concentrations, again no relative changes in GFR or ERPF were demonstrated. Under certain conditions, some workers have succeeded in demonstrating a CyA-induced effect upon renal haemodynamics (Weir et al 1989); the reasons for this discrepancy will be discussed later.
4.5 RENAL FUNCTIONAL RESERVE IN PATIENTS RECEIVING CYCLOSPORIN A

INTRODUCTION
This study was undertaken to investigate Renal Functional Reserve (RFR) in a group of renal allograft recipients (n = 9) who were taking CyA immunosuppressive therapy. Other workers have demonstrated that the transplanted kidney retains this capacity in patients taking azathioprine.

RESULTS
All 9 patients were able to ingest the high protein meal provided in less than 30 minutes and all were able to micturate at regular intervals throughout the study. Flow rates tended to be lower during the post-prandial period than in the fasting state, however, differences did not reach statistical significance (Appendix XI).

Patients who participated in this study were deliberately selected with a wide range of baseline renal function. Mean GFR prior to protein loading ranged from 20.9ml/min to 89.45ml/min with a mean value of 50.08ml/min. Corresponding values of ERPF ranged from 58.15ml/min to 386.1ml/min with a mean of 209.47ml/min.

Patient and transplant variables at the onset of the study are listed in Table 4.5.i.
<table>
<thead>
<tr>
<th></th>
<th>MEAN (+/-SEM)</th>
<th>RANGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient age</td>
<td>36.2 (5.07)</td>
<td>20-65 years</td>
</tr>
<tr>
<td>Donor age</td>
<td>32.2 (4.43)</td>
<td>15-53 years</td>
</tr>
<tr>
<td>Time since Tx</td>
<td>25.22 (2.88)</td>
<td>7-34 months</td>
</tr>
<tr>
<td>Cyclosporin A dose</td>
<td>6.39 (0.54)</td>
<td>4.5-10 mg/kg/d</td>
</tr>
<tr>
<td>Cyclosporin A trough level</td>
<td>198.11 (35.09)</td>
<td>95-444 ng/ml</td>
</tr>
<tr>
<td>Serum Creatinine level</td>
<td>165.9 (18.19)</td>
<td>123-279 µmol/l</td>
</tr>
<tr>
<td>24-hour Creatinine clearance</td>
<td>57.79 (8.00)</td>
<td>21-92 ml/min</td>
</tr>
<tr>
<td>Baseline GFR</td>
<td>50.09 (6.78)</td>
<td>20.9-89.45 ml/min</td>
</tr>
<tr>
<td>Baseline ERPF</td>
<td>209.48 (31.42)</td>
<td>58.15-386.1 ml/min</td>
</tr>
</tbody>
</table>

**Table 4.5.1** Patient and functional variables prior to the investigation of Renal Functional Reserve.
CyA trough levels were relatively low in these patients, 8 whole-blood trough levels falling below 225ng/ml. A value of 444ng/ml was recorded in one patient who was on a relatively higher CyA dose only 7 months post-transplantation.

Creatinine clearance measurements for the 24 hours prior to the study, tended to overestimate baseline GFR by a mean value of +17.4%, with individual variations ranging between -22.7% and +80% of the recorded GFR.

After measuring GFR and ERPF by isotope clearance for 3 separate one-hour intervals after the 93g oral protein load, no significant change in either parameter was detected (Fig 4.5.i).

By calculating individual changes in GFR and ERPF and expressing these as percentages of the mean baseline value for each patient (Fig 4.5.ii), it became apparent that most patients demonstrated a fall in renal plasma flow and filtration capacity immediately after the protein stimulus. This was then followed by a later recovery phase. Two patients did, however, demonstrate an increase in GFR and ERPF, a peak value of +20% being recorded in both parameters in one subject 2 hours post-prandially.
FIG. 4.5.i Mean values of GFR and ERPF (+/- 1SD) before and for 3 hours after the oral protein load.
FIG. 4.5.ii Percentage change in GFR and ERPF from a baseline value of 100% in each patient over the 3-hour study period.
The individual changes shown in GFR following the protein stimulus demonstrated no correlation with the baseline level of functional recorded in each patient. The raw data for individual changes in GFR throughout the period of the study are shown in Fig 4.5.iii.
FIG. 4.5.iii Crude data for individual changes in GFR relative to time after the oral protein stimulus.
CONCLUSION

It would appear that the capacity of the intra-renal circulation to increase in response to an oral protein load is absent in the majority of renal transplant recipients taking CyA immunosuppressive therapy. This may be related to the disturbance of intra-renal prostanoid synthesis induced by CyA.
CyA is the best available immunosuppressive agent at the present time. Its discovery and introduction into clinical practice just over ten years ago led to significantly improved patient and graft survival in all transplant units. One-year renal allograft survival rates were increased by at least 20% in most multicentre studies (Calne and Wood 1985, Canadian MCTS 1983). In addition, its more specific mode of action on T helper cells and interleukin-2 has reduced post-transplant morbidity. Not only have severe, life-threatening bacterial and fungal infections decreased but the incidence of malignancy, in particular lymphoma, has also shown improvement (Miach 1986, Green 1988). The specific immunological mechanism of action of CyA has also led to its successful use in a wide range of diseases with an autoimmune pathogenesis.

However, in the midst of these successes, the major problem of progressive nephrotoxicity seriously counterbalances the many advantages of this drug. It was soon realised that large doses of CyA induced an acute and probably reversible nephrotoxicity. First reports in man quickly demonstrated the acutely detrimental effects of this drug on renal function (Calne et al 1978). International reports have since demonstrated the chronic, progressive deterioration of graft survival seen in renal transplant recipients receiving long-term CyA (Land 1989). Functional deterioration in the native kidneys of patients receiving other transplants is sometimes even more rapid (Myers et al 1984, Greenberg et al 1987). However, many authors will dispute a chronic progressive effect of CyA on renal function (Hamilton et al 1982, Thiel et al 1985). Some believe that low dose CyA in combination with other therapies is a safer alternative (Post et al 1987). Consequently doses
were reduced in an attempt to find a therapeutic concentration which caused no nephrotoxicity. After several more years it became clear that no "therapeutic window" existed and that any dose could produce progressive, chronic deterioration of renal function. This was associated with characteristic but non-specific structural changes including striped interstitial fibrosis and glomerular arteriolopathy (Mihatsch et al 1988). The rate of onset of chronic CyA nephrotoxicity and hence gradual deterioration of renal function is not predictable and varies from patient to patient. It has also become apparent, however, that a renal allograft which has been subjected to any degree of damage, for example, acute cellular rejection or prolonged warm ischaemia, is more susceptible to the nephrotoxic effects of this drug (Land 1987).

Animal studies performed prior to human administration did not draw attention to the nephrotoxicity of CyA, as doses required to provide immunosuppression were not toxic in the species investigated. Once this side effect became appreciated, much work was undertaken in animal models where CyA nephrotoxicity could be induced by giving 5 or more times the human therapeutic dose. Animals then provided experimental models in which the toxicity of this drug could be investigated and hopefully reversed or ameliorated. For the last 10 years a vast amount of literature has been published on this subject. Several mechanisms of toxicity have been proposed and in some studies a degree of improvement in renal function has been achieved. However, one cannot always extrapolate directly from animal work to the human situation. For example, in the rat model, CyA stimulates the Renin-Angiotensin Aldosterone system (RAAS) producing elevated circulating concentrations of renin. This in turn induces increased production of angiotensin II, which is directly active on the efferent glomerular arteriole causing
vasoconstriction. In man, however, there is no comparable evidence for stimulation of the RAAS by CyA. Secondly, complex experimental physiological animal preparations are easily studied in the laboratory but similar models cannot be achieved in man. Animal preparations which involved the isolation of renal nerves and the measurement of sympathetic afferent and efferent impulses suggested that CyA might stimulate the autonomic nervous system. The human renal transplant remains denervated for at least several weeks after surgery, which by inference would contra-indicate a direct effect of CyA on the autonomic nervous system in the early post-operative period.

The majority of investigators would agree that intra-renal vasoconstriction is the primary mechanism responsible for CyA nephrotoxicity. This has been conclusively demonstrated in animal work (English et al 1987, Murray et al 1985) and supported strongly by human studies (Curtis et al 1986a), in particular conversion studies (Hall et al 1987). In addition, systemic hypertension is a recognised side effect of CyA therapy, which suggests a vasoconstrictive effect on extra-renal vessels.

Evidence for CyA having an additional tubulotoxic effect remains controversial. Whilst it is clear that the tubular handling of electrolytes, glucose and water is altered in the presence of CyA, some believe this to result from the ischaemic effect of intra-renal vasoconstriction, rather than a directly toxic effect upon tubular cell function. Direct toxicity has been demonstrated in cell culture but usually at much higher concentrations of CyA than would occur in vivo (Zoja et al 1986). The release of certain enzymes from renal tubular epithelial cells directly into the urine is a recognised indicator of tubular damage but is not a specific feature of CyA nephrotoxicity, also
being documented in renal transplant recipients receiving azathioprine immunotherapy (Finn and Gitelman 1984). CyA is known to induce glycosuria and all of the patients investigated in the present study demonstrated elevated levels of urinary glucose. Measurements of urinary glucose and NAG were expressed in units per mmol of creatinine excreted, which avoids errors resulting from variability in urinary concentration (Appendix IX). NAG excretion was also elevated in all patients as anticipated. The handling of other substances by the renal tubule may also be affected by a potential tubular toxin. In the patients studied, the urinary excretion of both sodium and potassium was low, the values measured for sodium falling just below a normal quoted range. Fractional excretion of sodium ($F_{\text{Na}}$) was elevated above normal in all patients at the onset of the trial suggesting impaired handling of sodium during CyA therapy. However, the mean value tended to fall to the normal range in both groups during the 3-month study period. In the placebo group, the decrease in $F_{\text{Na}}$ was much greater, reaching statistical significance ($p = 0.025$). This would suggest that despite continued CyA therapy, resorption of sodium may have increased during the study period, a finding which would be inconsistent with the chronic effects of a tubular toxin inducing irreversible structural damage. The much smaller decline in $F_{\text{Na}}$ in those treated with active drug was associated with a significant reduction in GFR and a fall in ERPF and could therefore result from a combination of decreased filtration and absolute resorption of sodium. Treatment with GR32191B in these patients may have affected tubular function via its effect on renal blood flow and GFR. These findings are consistent with those of Grace (1988) who felt that CyA produced changes consistent with a nephron which was functionally intact but under-perfused.
The movement of extra-cellular calcium ions is closely linked with both voluntary and smooth muscle activity and could therefore be closely linked with intra-renal vasoconstriction. The importance of calcium-ion movement in CyA nephrotoxicity is undergoing investigation in many centres at the present time.

Prostaglandins are powerful vasoactive autocoids; evidence has also proven a direct effect of CyA upon prostaglandin production in systems containing both renal and non-renal tissues. This study has attempted to address the problem of CyA-induced nephrotoxicity by manipulating the effects of certain prostaglandins in a group of renal transplant recipients.

Renal transplant patients must be considered as an unique group when compared with other people receiving CyA therapy. Each has only one functioning kidney and consequently maximum possible GFR will not exceed 80 to 85ml/min. Animal work has suggested that single kidney hypertrophy does not occur in the presence of CyA (Thiel 1986), whilst further animal studies also indicate that hyperfiltration may also not be possible (Provoost 1986). Consequently, in these patients the best GFR attainable may not exceed 60 or 70ml/min. Added to this is any further permanent loss of function which may have resulted from ischaemic damage, reflow perfusion, acute cellular rejection or acute toxic episodes occurring in the early post-transplant period. This relatively low level of function was adequately demonstrated by the total patient group investigated, in whom mean GFR was only of 45.2 (SD 12.3) ml/min with a range of 22.5 to 73.9 ml/min.

Prior to and even since the introduction of CyA as a leading immunosuppressive agent, the the majority of publications from both
transplant surgery and autoimmune therapy use SeCr measurements to indicate the level of renal function. Many references are made to "stable renal function" in cases where SeCr alone has been measured and has remained unchanged over a period of investigation. Some authors feel safer measuring $C_{1\text{Cr}}^q$ whilst others incorrectly refer to "GFR" when in fact only the clearance of creatinine has been measured.

Measurement of $C_{1\text{Cr}}^q$ from a 24-hour urine collection is a less reliable indicator of level of function, particularly in the renal transplant recipient. Urine collections made within the patients' own homes are subject to errors of sampling and timing. The values produced in normal people tend to over estimate GFR whilst in a population of renal transplant recipients, $C_{1\text{Cr}}^q$ measurements are even more inaccurate (Hood et al 1971, Danovich et al 1986, Wilkinson et al 1987). The discrepancy between $C_{1\text{Cr}}^q$ and GFR in the present study bore no relationship to the true level of function in individual patients.

SeCr concentration is affected by exercise and diet and is particularly related to lean-body mass. A manipulation of SeCr involving body weight might provide a useful modification of this concentration to equate with true GFR (Cockcroft and Gault 1976, Myara et al 1989). However, despite good correlation with isotopic estimations of GFR, the Gault and Cockcroft "GFR" value still varied from true GFR by between -20.6 and +110.03% when calculated from the first SeCr concentrations. When changes in GFR were investigated using this modification, a unit change in GFR was not paralleled by a comparable change in calculated "GFR".

Examining the SeCr v GFR curve (Fig 4.2.1, p 170), it is clear that kidney transplant patients fall well down the curve on the GFR ordinate. Up to 50% of a normal GFR can be lost before a concomitant rise in SeCr
is seen and consequently progressive deterioration from or soon after transplantation may not be immediately apparent. This is particularly true in patients with a true GFR above 45 ml/min. If the function of the transplanted kidney is good initially, early deterioration in graft function may not be detected. Chronic CyA nephrotoxicity is a slow but progressive disease (Land 1989) and clearly deterioration in the renal function of a transplant recipient will only become obvious from rising SeCr concentrations once the true GFR has fallen below a value of 45 ml/min.

Patients with other allografts and those with autoimmune disease may well have normal renal function and a GFR well above 100 ml/min at the onset of therapy. If clinicians continue to monitor renal function using SeCr concentrations alone, a large proportion of filtration capacity will be lost before a change in SeCr is recorded. Until that time, renal function will be regarded as "stable" and when a sudden rise in SeCr occurs, this will be misinterpreted as a sudden new deterioration in function.

If the early effects of CyA are functional and reversible, filtration capacity may be retained on cessation or modification of dosage. However, if SeCr alone is used to monitor renal function irreversible functional impairment will occur long before SeCr levels start to rise. At this stage CyA-induced structural damage may exist which will not respond to therapeutic manoeuvres. Acute dysfunctional episodes will still be detectable by a rapid rise in SeCr levels, however, chronic dysfunction will not.

Therefore, when monitoring long term trends in renal function, SeCr levels alone will not provide a reliable indicator of chronic,
progressive graft dysfunction. It would appear, therefore, that there exists no simple substitution for regular isotopic measurement of true GFR in all patients receiving CyA. This practice will obviously not avoid CyA nephrotoxicity but will draw attention to a regular decline in filtration capacity, hence enabling therapeutic manoeuvres to be implemented at an earlier stage, when it is to be hoped, an irreversible deterioration towards renal failure is not inevitable.

With the more recent introduction of techniques which involve only a single injection of isotope, the accurate measurement of both GFR and ERPF has become quick and simple to perform within the hospital ward. No particular precautions need be observed by the patient, as the radioactive dose received constitutes less than that from a single PA chest X-ray. (5MBq of $^{51}$Cr gives 0.15mSv to the kidney, 5MBq of $^{125}$I gives 0.05mSv; a CXR gives 1.5mSv to skin and 0.12mSv to bone marrow).

Prior to the introduction of the single-injection technique, isotope infusion methods had simplified the complex laboratory assays associated with measuring the clearances of both inulin and PAH. The single-injection method also avoids error introduced by the collection of timed urine samples. This technique can be repeated within a few days if necessary, the only precaution being the collection of a 0-minute blood sample prior to injection of isotopes. This is used to measure any remaining isotope activity and it is subtracted from each timed sample aspirated.

CyA has been the major immunosuppressive agent used in the Leicester Renal Transplant Unit since 1983. Most patients also receive prednisolone. Isotopic examinations of renal function have been performed on all patients at regular intervals after transplantation, since the beginning of 1988.
Plots of renal allograft survival, in Leicester, since the introduction of CyA in 1984, demonstrate both improved 1-year rates in addition to the continuous progressive loss of grafts which followed early success (Fig 4.1.i, p 157).

All patient groups clearly demonstrate the progressive deterioration of renal function associated with long-term CyA therapy (Fig 4.1.ii, p 158).

Early graft function interpreted from SeCr concentrations available during the post-transplant period was variable, with improved early function occurring in 1986 but deteriorating after that date. As has already been suggested (Section 1.3, p 5) improving results demonstrated from 1984 to 1986 may well indicate a "learning curve" following the introduction of CyA. Patients transplanted in 1986 demonstrate the best early and late function in terms of SeCr or GFR in combination with the most stable percentage graft survival.

As a result of the national shortage of suitable donor organs, it has been necessary recently to accept kidneys from older and less fit individuals in an attempt to meet the ever increasing demand. Consequently, despite both the experience gained in the handling of CyA and the improved, sensitive monitoring techniques now available, results are disappointing. Organs which are more susceptible to the nephrotoxic effects of CyA are being used more often, hence the improved results seen in 1986 cannot be maintained. Median donor age in 1986 was 30 years compared with 39 and 41.5 years for 1987 and 1988 respectively. Similarly, whilst only one-third of organs grafted in 1986 came from donors over 40 years of age, this figure had increased to 42.2% in 1987 and 55.6% by 1988.
From the isotope measurements of renal function performed on small groups of patients transplanted during each of the years 1986, 1987 and 1988, again superiority of function was demonstrated by those transplanted in 1986. This improved level of function was well maintained over their 3rd post-transplant year.

Easily the most disappointing results were demonstrated by patients transplanted during 1988, with rapid deterioration of function especially notable during the second half of the study period, despite attempts to alleviate this by reducing CyA dosages.

The kidney is known to have reduced blood flow and hence filtration capacity with increasing age, whilst an older kidney also has a greater risk of exposure to renal disease (Avendano and Novoa 1987). Consequently, an older kidney may be more sensitive and hence susceptible to the effects of ischaemia and in particular to the nephrotoxic effects of CyA. This theory is supported by the increased incidence of acute tubular necrosis immediately following transplantation in 1987 (21%) and 1988 (15.4%) compared with 1986 (10%). Increased sensitivity to the nephrotoxic effects of CyA may well account for the rapidly deteriorating renal function demonstrated during the first 12 months in patients transplanted during 1988. If this rapid rate of deterioration continues unabated, one might predict that a proportion of these organs may cease to function within a relatively short period of time.

As a result of the figures, in Leicester, much attention and effort has been applied to minimising the nephrotoxic effects of CyA. Maintenance doses of CyA were reduced in 1988 leading to lower overall whole-blood HPLC trough concentrations (Fig 4.1.vi, p 166). A local retrospective
study reported in 1987 (Feehally et al) and more recent work (McNally unpublished observations) have both demonstrated the beneficial effect of concomitant calcium-channel blockade in patients receiving CyA. As a result, most hypertensive patients in Leicester are treated with nifedipine or another calcium-channel antagonist and finally this investigation of a specific thromboxane receptor antagonist was undertaken.

To increase the source of more suitable younger donor organs, in 1989, the Leicester Renal Transplant Unit commenced a programme of education and information aimed at both the native and Asian communities in the hope of encouraging more living-related organ donation.

CyA treatment is known to increase the synthesis of the vasoconstrictor prostanoid TXA$_2$, both in animal models and man. The absence of elevated plasma concentrations in association with increased urinary excretion points to an intra-renal source for this substance. Sraer et al (1982) demonstrated the enzyme systems required for prostaglandin synthesis in cultured renal cells whilst these preparations can also produce small quantities of TXA$_2$ spontaneously (Scharschmidt and Dunn 1983). In vitro experimental evidence suggests that CyA stimulates renal tissue to synthesise TXA$_2$. In addition, there is evidence for a small contribution provided by platelets circulating through the kidney and even perhaps from macrophages within the kidney (Benigni et al 1988a and b). The TXA$_2$ produced activates platelets locally, stimulating further TXA$_2$ production and release leading to a cascade phenomenon. The vasoconstrictory response to increased TXA$_2$ production may be partially responsible for the nephrotoxicity of CyA.

In tissue and cell culture models containing vascular endothelium, CyA
has an additional effect, appearing to inhibit the synthesis of the vasodilating prostanoid $\text{PGI}_2$ (Neild et al 1983, Brown and Neild 1987). In the intact animal, this inhibitory effect of CyA is more difficult to demonstrate.

Studies have attempted to reduce $\text{TXA}_2$ levels by blockade of total prostaglandin production with non-steroidal anti-inflammatory drugs (NSAID's) which inhibit activity of the enzyme cyclo-oxygenase. However, these drugs also abolish the production of vasodilatatory prostanoids, most notably $\text{PGI}_2$ and $\text{PGE}_2$. In people with an element of renal failure, treatment with these drugs is associated with deterioration in renal function. Patients with renal disease maintain their renal function by the constant influence of a vasodilatatory prostaglandin "drive" (Brenner and Rector 1982).

Selective inhibition of the enzyme thromboxane synthase would not decrease and may even enhance the production of vasodilatatory prostanoids by redirecting accumulating endoperoxides via the alternative limb of the prostaglandin synthetic pathway (Fig 5.i). However, endoperoxides are powerful $\text{TXA}_2$ agonists producing vasoconstriction in their own right. Any improvement seen in renal function when giving a TSI in combination with CyA, therefore, tends to be incomplete (Perico et al 1986b). The natural progression of this therapeutic approach to CyA nephrotoxicity is the use of a drug which will selectively antagonise the $\text{TXA}_2$ receptor.

In this study renal function was measured accurately using isotope clearance methods which provide the present day "gold standard" for the measurement of GFR and ERPF. The results obtained were compared with synchronous SeCr and $\text{Cl}_C$ measurements.
FIG. 5.1 The Prostaglandin Synthetic Pathway.
In order to investigate the biochemical effects of the TXA$_2$ receptor antagonist, it was important to demonstrate elevated urinary TXB$_2$ levels in the transplant patients taking CyA. The urinary concentration of 6kPGF$_{1a}$ was also measured as an indicator of PGI$_2$ synthesis. These measurements were repeated after 3 months of treatment with either active drug or placebo.

GR32191B, manufactured by Glaxo Group Research was tested extensively prior to clinical use and demonstrated to be a competitive, specific TXA$_2$ receptor antagonist. It had virtually no side effects, no agonist properties and no significant effect on other enzymes in the prostaglandin synthetic pathway in the preparations investigated (Humphrey et al. 1990). The platelet and smooth muscle activity produced by a range of selective thromboxane receptor agonists was blocked both in vivo and in vitro by this compound (Lumley et al. 1988, 1989). When given to normal healthy volunteers in a dose of 40mg twice daily, GR32191B achieved adequate blood concentrations to inhibit U46619-induced platelet aggregation (Thomas et al. 1987). On the basis of this result, 40mg of drug or placebo tablets were given twice daily to a group of renal transplant recipients for a 3-month period.

The patients were allocated at random to either drug or placebo on a double-blind basis, which produced two comparable groups of patients when the code was broken. In order to reduce variations to a minimum in the patients who agreed to participate, none was less than 6 weeks nor more than 2 years post-transplantation. In addition, all patients were also taking nifedipine, a calcium-channel blocker, for the treatment of hypertension. The treatment groups were further stratified according to the individual time period since transplantation. Unfortunately, the number of patients within each timed group was small. Statistical
significance indicated by these small groups must therefore be viewed with caution.

Several other trials involving the use of GR32191B have been completed. The majority were performed in patients with vasculopathic and platelet-related diseases or undergoing surgery for such conditions. In addition, nephrotoxicity in heart and liver transplant recipients was also studied. From the data available in January 1990, the overall incidence of malignancy was low and similar in placebo-treated (0.34%) and drug-treated (0.42%) participants. 10 deaths had occurred in patients taking GR32191B compared with 11 on placebo. As the majority of patients already suffered severe cardiological or vascular disease, no causal relationship was indicated.

From the platelet-blocking activity of this drug, problems with bleeding were anticipated. Other studies reported 3 fatalities associated with haemorrhage and one patient in the present study also developed a similar but non-fatal problem following a transplant biopsy. In addition, however, in the present study, 7 patients underwent minor surgical procedures (including removal of a CAPD catheter, liver biopsy and closure of an arterio-venous fistula) without problem. It would appear that the risk of haemorrhage is greater with major surgery or bleeding from relatively large vessels.

By the nature of the selection criteria for this trial, each timed group contained only patients with relatively good renal function. All patients recruited had a SeCr of less than 300umol/l. It has been stated (Land 1987) that any event which brings about renal damage can increase susceptibility to CyA toxicity and therefore lead to a more rapid rate of deterioration of renal function. Consequently, patients
recruited to Group 4 had suffered only 2 (placebo group) or 0 (drug group) rejections since transplantation. Other patients transplanted during the same time period who suffered more episodes of rejection may have either lost their transplants or developed functional deterioration to a level which excluded them from the trial. This "positive selection" partially explains why function measured as GFR is progressively better through the Groups described (the number of rejections recorded in each group is in parentheses).

<table>
<thead>
<tr>
<th>date of Tx</th>
<th>1986</th>
<th>1987</th>
<th>1988</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>4</td>
<td>2+3</td>
<td>1</td>
</tr>
<tr>
<td>PLACEBO</td>
<td>51.3 (2)</td>
<td>44.8 (5)</td>
<td>43.8 (9)</td>
</tr>
<tr>
<td>DRUG</td>
<td>45.4 (0)</td>
<td>47.3 (4)</td>
<td>46.7 (2)</td>
</tr>
</tbody>
</table>

**Table 5.i** Mean GFR demonstrated by each timed group.

Despite some small significant changes which fell within laboratory normal ranges, GR32191B did not demonstrate any marked effect upon haematology, liver and bone biochemistry or urinalysis. Decrease in proteinuria and increase in serum total protein measurements probably reflected recovery from ischaemic damage which the transplanted kidney suffers during harvesting and storage in addition to dietary changes. MCV decreased slightly with time and might reflect the high incidence of upper gastrointestinal mucosal lesions associated with steroid therapy and requiring H₂-receptor blocking therapy in many of these patients.

The results obtained from patients who received placebo should provide a guide to the expected rate of change of function demonstrated by a population of renal transplant recipients over a 3-month period. This
is a relatively short time interval and when the results were analysed together, no significant changes nor trends were demonstrated.

If one examined the smaller individual groups, trends could be seen. Group 1 patients had suffered a total of 9 episodes of acute cellular rejection prior to entry into the trial. Over the short study period these patients demonstrated rapid deterioration of both GFR and ERPF, which was not matched by an appropriate change in SeCr. The other groups had more stable values of GFR and ERPF although again SeCr concentrations did not always reflect this stability.

It is interesting that RVR recorded in each individual placebo-treated group demonstrated an improvement over the period of investigation. At the beginning of the trial the highest RVR was recorded in Group 1 which had suffered the most rejection episodes, whilst the lowest RVR was recorded in Group 4.

<table>
<thead>
<tr>
<th></th>
<th>PRE-</th>
<th>POST-PLACEBO</th>
<th>REJECTIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>52.6</td>
<td>51.9</td>
<td>9</td>
</tr>
<tr>
<td>Group 2+3</td>
<td>50.6</td>
<td>46.5</td>
<td>5</td>
</tr>
<tr>
<td>Group 4</td>
<td>48.55</td>
<td>42.81</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 5.ii Trends in RVR demonstrated by patients who received placebo.

The calculation of filtration fraction (FF = GFR/ERPF) avoids variations which result from day to day changes in ERPF. In the placebo groups, mean values of FF varied by less than 0.2% from the original measurements after the 3-month investigation period. This indicated a constant relationship between GFR and renal blood flow throughout the study period.
In conclusion, control patients showed remarkable stability in the parameters used to monitor renal function over the 3-month period of investigation.

In contrast, every individual group receiving GR32191B demonstrated deteriorating function, in which the decrease in GFR achieved significance when patients were analysed collectively. This occurred despite the fact that these patients had previously suffered fewer early rejection episodes than the placebo group and as a result might be less sensitive to the nephrotoxic effects of CyA. Secondly, as evidence suggests TXA$_2$ may be a possible mediator of the nephrotoxic effect of CyA, treatment with GR32191B, a competitive TXA$_2$ receptor antagonist, would be expected to improve renal function. If TXA$_2$ were not involved in CyA nephrotoxicity, one would anticipate similar results to those demonstrated by the placebo group, ie. no changes in overall renal function. However, despite anticipating improvement in renal function from the predicted theoretical basis of this work, a significant decrease in GFR occurred in association with deteriorations in both ERPF and SeCr.

As in the placebo group 1, a moderate but non-significant drop in mean GFR from 46.7 to 37.9 ml/min was noted. Significant deteriorations in both ERPF and SeCr were recorded, however, in this group. Patients in the other groups also suffered decrements in GFR which achieved significance when results were combined. Again, changes seen in SeCr levels in these groups bore little relationship to the changes in GFR.

In complete contrast to the placebo group, RVR increased in all groups during the study period although never reaching significance. The greatest increase in RVR was demonstrated in the group which had
experienced the most rejections.

<table>
<thead>
<tr>
<th></th>
<th>PRE-</th>
<th>POST-GR32191B</th>
<th>REJECTIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>41.2</td>
<td>46.5</td>
<td>2</td>
</tr>
<tr>
<td>Group 2+3</td>
<td>47.5</td>
<td>55.52</td>
<td>4</td>
</tr>
<tr>
<td>Group 4</td>
<td>50.1</td>
<td>53.7</td>
<td>0</td>
</tr>
</tbody>
</table>

dyne sec/cm²

Table 5.iii Trends in RVR demonstrated by patients who received GR32191B.

Filtration fraction of patients who received GR32191B decreased by a mean value of 1.19%, a decrease of more than 1% being recorded in both Groups (2+3) and 4. This indicates that the measured fall in ERPF was accompanied by a relatively greater deterioration in GFR.

These results suggest that treatment with GR32191B has firstly brought about an increase in intra-renal vasoconstriction, demonstrated by falling ERPF and rising RVR. However, the relatively greater fall in GFR suggested by the change in FF indicates that an additional factor must have decreased the ultra-filtration coefficient further.

The tendency for GFR to decrease without a parallel fall in ERPF and for RVR to increase without an equivalent trend in systemic BP, are effects very similar to those which occur as a result of CyA therapy alone. A decrease in GFR which was not proportional to the fall in ERPF was reported in both animal (Perico et al 1986b) and human studies (Tomlanovich et al 1986, Weir et al 1989). It is believed that in addition to an effect on the vascular smooth muscle of the afferent arteriole decreasing renal blood flow, CyA also has an indirect contractile effect upon the mesangial cells of the glomerulus, reducing the surface area available for filtration. TXA₂ production induced by
CyA therapy is thought to be responsible for both effects.

In the patients investigated, however, these changes occurred despite apparent specific TXA$_2$ receptor blockade. Thus GR32191B appears to have worsened rather than improved renal function whilst the mechanism by which function has deteriorated resembles that produced by CyA therapy.

To elucidate further, the measurement of prostanoid metabolites was undertaken. This study was confined to the investigation and measurement of TXA$_2$ and PGI$_2$ as the activity of these two substances seems closely linked with vascular smooth muscle reactivity and with the nephrotoxicity of CyA. As both substances are highly reactive and unstable, they were detected and measured as the more stable hydration products, TXB$_2$ and 6kPGF$_{1a}$ respectively.

The same RIA was used to measure plasma and urinary concentrations of each prostanoid metabolite and a solid phase extraction step was employed with both urine and plasma samples.

Prostaglandins are metabolised close to their site of synthesis. It was originally believed that TXB$_2$ and 6kPGF$_{1a}$ were produced within the kidney and therefore their urinary concentration closely reflected the intra-renal synthesis of TXA$_2$ and PGI$_2$. In comparison, the concentration of 2,3-dinor metabolites found in urine was thought to reflect the systemic synthesis of TXA$_2$ and PGI$_2$. Experimental evidence exists which supports this theory of different metabolites originating from differing sources. For example, in renal lupus erythematosus, whilst urinary TXB$_2$ excretion was elevated, 2,3-dinor TXB$_2$ was not (Patrono et al 1985). Similarly, sulindac is a non-steroidal anti-inflammatory drug (NSAID), thought to spare the renal cyclo-oxygenase enzyme system. In experimental work, it reduced the excretion of 2,3-
dinor TXB₂ (systemic) but not TXB₂ (renal). Ibuprofen, another NSAID, has no selective renal sparing effect and reduced the excretion of both forms of TXB₂ (Cibattoni et al 1984). On the contrary, however, other studies have demonstrated elevated urinary concentrations of both metabolites apparently originating from a single obvious source. Increased urinary excretion of both 2,3-dinor TXB₂ and TXB₂ was noted in syndromes associated with platelet activation (Catella et al 1986) and in the urine of rats given large doses of CyA (Benigni et al 1988b).

It has become apparent over the last decade that several sources of error can arise when attempting to measure prostaglandin concentrations, particularly in plasma specimens. It is easy to produce artefactually high concentrations unless strict methods of collection, separation and storage are observed. If multiple extraction and purification steps are undertaken prior to final assay, the concentration of a specific prostanoid is markedly reduced. Finally, despite the manufacturers' claims, antibodies in RIA kits may not be particularly specific. As the Amersham I²⁻¹²⁵ assay kits used in this study did not state a cross-reactivity with the 2,3-dinor forms of TXB₂ and 6kPGF₁α, at my request, they arranged to investigate this problem. A figure of 38.4% was detected for the cross-reactivity between the antibody in the TXB₂ assay kit and 2,3-dinor TXB₂ and 23.8% was quoted for the similar cross-reactivity between the 2,3-dinor form of 6kPGF₁α and the antibody to 6kPGF₁α.

In a recent study, normal controls produced urinary TXB₂ at a mean rate of 39.9ng/hr, consistent with a daily production of less than 1000ng/24hours (Weir et al 1989). All the patients investigated in the present trial excreted almost twice that amount daily, with a mean hourly excretion of 81.21 (SD 37.93) ng/hr. The excretion of 6kPGF₁α
fell within the normal range quoted by the assaying laboratory at 54.78 (SD 21.53) ng/hr (Appendix VII), but well below the hourly excretion measured in normal subjects by Weir et al (1989). These results demonstrated the elevated TXA$_2$ production characteristic of CyA therapy and may also support a possible inhibitory effect of CyA on PGI$_2$ synthesis described by some authors (Kho et al 1988, Deray et al 1988). To date, no feedback mechanisms have been described in the prostanoid synthetic pathway and as predicted, receptor blockade had no significant effect on TXB$_2$ excretion when compared with control values. In addition, as the prostacyclin synthetic pathway would also not be affected by a thromboxane receptor antagonist, no change in 6kPGF$_{1a}$ excretion was detected either after treatment.

After 3 months of placebo, excretion of TXB$_2$ increased in all groups and was significant when the results were summated. After 3 months of treatment with GR32191B, a similar increase in TXB$_2$ excretion occurred. This result suggests that TXA$_2$ receptor blockade has little or no effect upon the intra-renal synthesis or urinary excretion of thromboxane A$_2$. The urinary excretion of 6kPGF$_{1a}$ did not alter significantly over the period of investigation in either group, however, both demonstrated a tendency for the excretion of 6kPGF$_{1a}$ to decrease.

Changes in urinary or plasma prostanoid concentrations were identified by comparing the results obtained with values collected from a group of healthy volunteers, assayed under similar conditions. However, in light of the available data it was not possible to identify accurately the source of the prostanoid measured nor its specific form. In conclusion, a 3-month period of treatment with a TXA$_2$ receptor antagonist did not alter the total renal excretion of any of these prostanoid metabolites when compared with a placebo group. If one believes that the
metabolites measured in urine are of renal origin, GR32191B did not appear to have any effect upon renal TXA$_2$ or PGI$_2$ synthesis.

It must be appreciated that the changes in circulating 6kPGF$_{1a}$ detected in this study suffer drawbacks from the method of extraction and non-specificity of the assay employed in addition to the small sample size. It would be useful to repeat the measurements using a more stringent plasma separation technique and an assay specific for the 11-dehydro-6kPGF$_{1a}$ metabolite which is thought to be more stable and less liable to artefactual stimulation. However, despite these drawbacks, changes in the plasma concentrations of some prostanoid metabolites were demonstrated, using the methods described. Precautions taken over collection, processing, storage and assay of plasma samples appeared to be adequate and did not appear to include undue artefact resulting from platelet activation or endothelial trauma. All concentrations measured from plasma fell within normal ranges quoted by other workers using similar techniques. That is 100 to 200pg/ml for circulating TXB$_2$ (Catella et al 1986) and 1.5 to 3.5pg/ml for 6kPGF$_{1a}$ (Whiting plasma from 10 normal subjects, assayed in the same manner - unpublished observations).

Plasma concentrations of TXB$_2$ remained constant in the drug and placebo groups both before and after the 3 months of medication. However, 6kPGF$_{1a}$ concentration in plasma demonstrated a significant decrease, by 30% of pre-treatment levels after 3 months of therapy with GR32191B. No change in 6kPGF$_{1a}$ concentrations was noted in the control group.

In attempting to understand the effect of GR32191B in this group of renal transplant recipients, several factors have to be considered. Firstly, all the parameters measured have wide variation and most have
been examined in small patient numbers. With the many statistical analyses being performed, an occasional statistical association will occur which results from chance and does not truly represent an important change. Secondly, the mechanisms of action and interactions of the drugs discussed are far from being fully understood and thus a certain amount of conjecture has to be applied.

When one tries to understand why GR32191B did not improve renal function, it is important to know if the bioavailability of this compound was affected by any other medications being taken by most patients concurrently. As all patients took the drug at approximately the same time as their CyA each day, an interaction at the level of absorption is a possibility. CyA levels did not vary throughout the trial and thus CyA absorption did not appear to be affected. However, CyA might have affected the absorption of GR32191B. More importantly, GR32191B is broken down within the liver, the site of many major metabolic activities and in particular the degradation of CyA and steroids. As the blood from the gastrointestinal tract passes immediately to the liver in the portal venous system, GR32191B metabolism could be either enhanced by a previously stimulated enzyme system or blocked by another drug which shares the same metabolic pathway. The plasma assay for GR32191B involves a complex HPLC method and is only available at Glaxo Group Research Laboratories in Ware, Hertfordshire. In studies of normal volunteers, 40mg of GR32191 given twice daily produced adequate TXA2 receptor blockade (Thomas et al 1987) and for this reason and also because Glaxo felt it was unnecessary, levels were not measured in these patients.

Another possible explanation for the absence of beneficial effect of GR32191B arises from the timing of treatment. All patients receiving...
GR32191B were at least 6 weeks post-transplantation at the time of recruitment, the great majority being 6 months or more since surgery. From previous work, the vasoconstrictive nephrotoxic effect of CyA is seen immediately following a large single dose in animals and as early as 2 days after its therapeutic use in transplant recipients (Greenberg et al 1987, Jones et al 1988). Most studies demonstrate some improvement in renal function on conversion to alternative therapy.

After a single dose, complete reversal of the nephrotoxic effects may be possible, however, after a longer period of treatment with CyA, function may never return to normal (Hamilton et al 1982, Hunt et al 1987). Therefore, if TXA₂ is the active mediator of CyA nephrotoxicity, GR32191B should ideally be given to the patient from the first dose of CyA. Transplanted kidneys have suffered several "insults" prior to the onset of function and are probably more susceptible, as a result, to the toxic effects of CyA. Consequently by the 6th post-operative week, irreversible changes may already have commenced leaving less "reversible" function available for "release". Despite this, conversion studies performed 3 or more months post-operatively still demonstrate improvement in function. Therefore, if TXA₂ is an important mediator of CyA-induced vasoconstriction, giving GR32191B from the 6th week should still have produced a measurable improvement in function, perhaps demonstrated most clearly in Group 1 patients. These, however, demonstrated a significant decrease in both ERPF and SeCr associated with deterioration of both GFR and Clᵣ. Therefore, the timing of administration of GR32191B did not appear to be an important factor in the results produced.

A better explanation for the negative effect of GR32191B lies in the fact that all patients studied were also receiving a calcium-channel...
blocker: CyA therapy is known to be associated with an increased incidence of hypertension in allograft recipients. In a retrospective study performed in Leicester, Feehally et al (1987) also showed that renal allograft patients receiving nifedipine for the treatment of their hypertension had better renal function as assessed by Scr levels, than those receiving other antihypertensive agents. The Scr levels recorded were comparable with those found in a normotensive "control" group of allograft recipients also receiving CyA. More recently, a small group (n=10) of normotensive renal transplant patients receiving CyA were treated with nifedipine for a period of 1 month (McNally unpublished observations). A significant increase in GFR occurred whilst the measured increase in ERPF did not reach significance. A control group of patients treated with the immunosuppressive drug azathioprine, demonstrated no changes in GFR or ERPF after similar treatment with nifedipine. It is interesting that the mean pre-treatment GFR in the CyA patients was 38ml/min and this value increased to 44ml/min after nifedipine. The overall mean GFR of the patients in the GR32191B study was 45ml/min prior to therapy. Most of these patients had taken nifedipine at least since transplantation. In order to obtain 10 patients who were on CyA but not nifedipine, patients transplanted as long as 5 years were included in this study. Despite this long exposure to CyA, improvement in function still occurred.

Patients taking nifedipine were selected for the GR32191B trial for two reasons: firstly, to avoid any variation which could be encountered if this drug were commenced during the period of investigation; secondly, because the number of patients who satisfied the other trial criteria and were not receiving nifedipine was extremely small.

Calcium ions are intimately involved in the intra-cellular mechanism of
muscle contraction, regardless of the chemical instigator of that contraction. Nifedipine specifically inhibits calcium entry to smooth and cardiac muscle cells producing relaxation in response to stimulation. This effect only occurs when the muscle is already in tone; no effect is seen on blood pressure if normotensive people take therapeutic doses of nifedipine. The individual calcium-channel antagonists appear to act at different sites and Loutzenheiser and Epstein (1987) postulated a heterogeneity of calcium channels to explain the differing effects of individual drugs. Organic calcium-channel blockers also appear only to reverse the effects of an agonist which causes vasoconstriction of the afferent arteriole. Thus nifedipine will improve both KCl- and U44069- (a thromboxane-mimetic) induced decreases in GFR and RBF but has less effect on nor-adrenalin- and angiotensin II-induced constriction of the efferent arteriole.

In addition, it has also been suggested that decreased calcium concentrations might inhibit renal prostaglandin production (Kirschenbaum and Chaudhari 1986, Bunke et al 1988).

Calcium ions are also the intra-cellular mediators of TXA2 activity. Stimulation of a muscle cell by TXA2 leads to a rise in intra-cellular calcium concentration. PGI2 induces a rise in intra-cellular cAMP concentration, which in turn blocks calcium ion movement from extra-cellular to intra-cellular sites. Therefore calcium-channel blockade will actively inhibit the effects of TXA2 and passively enhance the actions of PGI2. In vitro, calcium-channel blockers inhibit both TXA2 mimetic-induced constriction of the afferent arterioles and mesangial cell contraction, which also relies on the entry of calcium ions (Loutzenheiser and Epstein 1987).
Therefore, the fact that all the patients studied were also receiving nifedipine treatment provides a reasonable explanation of why renal function did not improve in the patients selected to receive GR32191B. However, this does not explain the significant deterioration of renal function demonstrated by those receiving active drug.

As previously mentioned, the measurement of plasma prostanoids has drawbacks as the concentrations measured by RIA may be relatively large but can be vastly reduced by repeated extraction and purification of samples prior to assay, some workers quoting much lower normal ranges. Despite these drawbacks, the results produced in this study compared well with a range produced from normal volunteers and measured by the same technique in the same laboratory. As the assay was performed prior to access to the code, two unequal groups were produced, plasma concentrations from only 7 patients receiving GR32191B being measured. As stated, TXB$_2$ and 6kPGF$_{1a}$ concentrations remained within normal quoted ranges both before and after the treatment period. No change occurred in the circulating venous concentration of TXB$_2$ in either group of patients. However, patients who received GR32191B demonstrated a significant decrease in circulating concentrations of measurable 6kPGF$_{1a}$ after therapy. Both mean and median concentrations decreased by almost 30% of the baseline value. When overall plasma prostanoid "activity" was considered (6kPGF$_{1a}$/TXB$_2$), there appeared to be an association between increased vasoconstrictive activity and treatment with GR32191B.

GR32191B by its antagonism of TXA$_2$, also inhibits platelet reactivity, preventing aggregation and adhesion and subsequently increasing the bleeding time. This action has been demonstrated in normal volunteers (Maconochie et al 1988). TXA$_2$ concentrations were not previously measured during this inhibition, whilst the patients studied here
demonstrated no changes in circulating TXB$_2$ concentrations. Nifedipine also appears to have an effect on platelet activity in the presence of CyA but only completely inhibits aggregation at trough levels (Grace et al 1987). CyA is known to cause endothelial damage and this in turn stimulates platelet activation leading to an increased thrombotic tendency. In the presence of the TXA$_2$ receptor antagonist, GR32191B, platelet activation and aggregation will be inhibited and thus adhesion to endothelial cells will similarly be reduced. Endothelial cells will be less stimulated and in this situation, one could postulate that the production of PGI$_2$ and hence systemic concentration of its metabolites, would be decreased. This hypothesis assumes that adequate circulating concentrations of GR32191B were achieved in the patients investigated and does not determine the specific metabolite of PGI$_2$ being measured. It also seems reasonable to assume that if the concentration of a substance detected circulating in venous plasma changes, the physiological effect might be experienced within any organ.

This hypothesis may provide an explanation for the significant reduction in plasma 6kPGF$_1\alpha$ concentrations demonstrated by patients receiving GR32191B. As the circulating levels of 6kPGF$_1\alpha$ dramatically decreased by almost 30%, any prostacyclin-maintained vasodilatation within the kidney might be reduced as a result. It is known that the kidneys of patients with renal dysfunction appear to work under the influence of a PGI$_2$ "drive" such that general prostaglandin inhibition, produced by giving a NSAID leads to a decrease in function. Prostacyclin is believed to be produced as a regulator in response to any stimulus which causes intra-renal vasoconstriction. If circulating levels of PGI$_2$ are markedly reduced, loss of a proportion of the PGI$_2$-induced intra-renal vasodilatation may follow.
Mesangial cells are also sensitive to the actions of vasodilatory prostaglandins. In rat glomerular culture, Scharschmidt et al (1983, 1986) demonstrated an increase in glomerular planar surface area in response to PGE$_2$, the major renal vasodilatory prostanoid in this animal. Consequently in the hypothesis suggested in man, decreasing the circulating concentration of PGI$_2$ would also induce mesangial cell contraction. Therefore, the overall effect would be a combination of increased vasoconstriction reducing ERPF and GFR, and an increase in mesangial cell tone, reducing the surface area for filtration, which would lead to a further reduction in GFR.

As no change was detected in either the urinary or plasma concentration of TXB$_2$ in the presence of the TXA$_2$ receptor antagonist, the synthetic pathway for TXA$_2$ production does not appear to be under a feedback control mechanism.

This explanation for the results obtained may be far too simplistic, however, the association of deteriorating renal function with a significant decrease in circulating levels of a major prostacyclin metabolite cannot be ignored. In addition, the known physiological actions of PGI$_2$ appear to fit well with the functional changes recorded.

With the intention of investigating the effects of GR32191B in patients receiving CyA but not nifedipine, an attempt was made to produce a human model of acute CyA nephrotoxicity.

From the work performed in rats it would appear that a single large intravenous dose of CyA produces a rapid although transient decrease in RBF and GFR. Paller et al (1985) found that 20mg/kg iv produced this effect but 10mg/kg iv was insufficient. The immediate effect of a large oral dose is not reported. English et al (1987) gave 50mg/kg orally to
rats and measured function on day 3. By this time a significant fall in
the clearance of inulin was detected in association with CyA whole-blood
RIA trough levels of between 3000 and 4000ng/ml. The reported changes
in afferent arteriole diameter were not apparent at this time, narrowing
only becoming significant on day 7. This work suggests that CyA may
have an effect on GFR and the ultra-filtration coefficient which
precedes any vasoconstriction of the intra-renal vasculature.

In man, fewer studies have described the immediate response of renal
function to an oral dose of CyA, although evidence for the reduction in
GFR and RBF after several days or weeks of treatment is widely reported.

The renal transplant recipients in this particular study received a
relatively low dose of CyA superimposed upon a background of between 6
and 22 months of chronic dosing with CyA. In the 4 patients studied,
individual therapeutic doses produced relatively low CyA peak blood
levels and no significant functional responses were observed.

The same procedure was then repeated by giving a much larger oral dose
of CyA to a group of patients with normal renal function who were about
to commence CyA for the treatment of psoriasis (Heule et al 1988).
Using 12mg/kg it was hoped to demonstrate the acute reduction in GFR and
decrease in ERPF seen both in animals (Paller and Murray 1985) and in
man (Weir et al 1989) and then to investigate the effects of GR32191B on
this model. However, despite achieving high peak blood concentrations,
again no acute effects on RBF and GFR were demonstrated.

Hauser et al (1988) measured the clearance of inulin and PAH in a group
of stable renal transplant recipients who had previously taken
azathioprine and prednisolone post-operatively. Single clearances were
measured over a 3-hour period. Significant decreases in both GFR and
ERPF were recorded following a single first oral dose of 10mg/kg. These patients achieved maximum blood levels of 1957 (SD 773) ng/ml at 3 hours. Weir et al (1989) gave an infusion of 4mg/kg to 8 normal women and achieved mean peak blood levels of 1216, 1431 and 1680ng/ml at the 2nd, 4th and 6th hour respectively. No significant change in RBF was recorded but GFR decreased significantly.

Despite receiving a relatively large oral dose of 12mg/kg of CyA, whole-blood concentrations achieved in this study did not parallel those recorded by Hauser et al (1988) or Weir et al (1989). These patients demonstrated no trends in GFR or ERPF, whilst the relationship between these two variables, filtration fraction, remained constant. Absorption of the oral dose of CyA given was inadequate to produce blood levels which would significantly alter renal function. Mean peak concentration achieved was 967.5 (SD 185.89) ng/ml. It is difficult to understand how much higher blood concentrations were achieved by Hauser and colleagues, despite giving only 10mg/kg and also using a monoclonal RIA. The bioavailability of CyA is not known to be increased by renal transplantation, although may be increased after taking CyA for some time.

It is most likely that the normal healthy kidney is far less susceptible to the immediate nephrotoxic effects of CyA at this dose. After a large intravenous infusion, which avoids immediate hepatic metabolism, higher blood levels were achieved and an immediate functional effect was demonstrated by Weir and co-workers (1989).

In renal transplantation lower doses of CyA are now widely employed. It is possible that the haemodynamic effect of CyA in renal allograft recipients is more marked. It has already been stated that a kidney
which has suffered ischaemic damage may be more susceptible to the nephrotoxic effects of CyA. In an elaborate animal study which supports this theory, Provoost (1986) demonstrated a moderate and completely reversible decrease in the renal function of rats receiving 15mg/kg of CyA per day intra-muscularly for a period of 4 weeks. However, rats who had suffered both unilateral nephrectomy and ischaemic damage to the remnant kidney were not able to reach control values of GFR on cessation of CyA and had obviously suffered a permanent loss of function as a result of only short term therapy.

Therefore although the renal allograft may be more susceptible to the nephrotoxic effects of even low doses of CyA, the four patients investigated may already be suffering maximum vasoconstriction as a result of high tissue concentrations from prolonged administration.

In the dermatology patients, the lack of acute vasoconstriction in response to a large single dose of CyA is more likely to have resulted from a combination of inadequate dosing and the presence of normal healthy native kidneys.

Following unilateral nephrectomy, a single kidney has the potential to hyperfiltrate immediately and to hypertrophy over the following months. This kidney also retains a Renal Functional Reserve (RFR) over and above the other capacities, which can be called upon in times of renal disease associated with loss of function. Animal work has demonstrated that CyA inhibits the first two responses (Thiel 1985) whilst Provoost (1986) has demonstrated in the rat, that a proportion of hyperfiltration can be re-acquired soon after cessation of CyA therapy.

Patients with a renal allograft taking azathioprine retained their RFR when stimulated by an intravenous amino acid infusion (Cairns et al
or an oral protein load (McNally unpublished observations). Cairns and co-workers (1989) also demonstrated no RFR in a small group of patients taking CyA when given intravenous protein. However, oral protein is thought to be a better stimulus of RFR than intravenous amino acids (Rodriguez-Iturbe et al 1988) whilst dietetic protein preparations such as Fortimel appear to have no effect (Dhaene et al 1987). Protein of meat origin is thought to be the best stimulus of RFR, producing a "dose response" up to a maximum dose of 150g (Amore et al 1988).

The important factor in the protein source appears to be the content of the neutral amino acids glycine, alanine and the basic amino acid arginine. The branched-chain amino acids valine, isoleucine and leucine produced no significant changes in GFR (Clarris-Appiani et al 1988). A meal containing 93g of protein and consisting of 5983mg of glycine and 6809mg of arginine, compared with 5600mg and 6720mg of the same amino acids respectively in a 16oz. cooked steak, was ingested by each patient within a 20- to 30-minute period. Despite this relatively large stimulus, no significant overall change in either GFR or ERPF was recorded. Two patients demonstrated a small non-significant response but this was absent in the majority.

Previous reports have suggested that patients with renal dysfunction demonstrate a RFR which is reduced in proportion to their baseline level of function, no reserve being demonstrated at a GFR below either 40ml/min or 30ml/min in different studies (Bosch et al 1984, Ter Wee et al 1986). Patients in this study were deliberately selected with a wide range of GFR previously measured accurately by $^{51}$Cr EDTA isotope clearance. In 6 of the patients investigated, baseline GFR exceeded 40ml/min and thus a measurable increase in response to an oral protein load might be anticipated. However, no correlation was demonstrated
between the baseline level of renal function and any changes observed. All patients in this study were taking nifedipine for the treatment of hypertension. It is known that antihypertensive drugs can modify renal blood flow, thus it is possible that calcium-channel blockade might account for the absence of RFR. However, in the study by Cairns et al (1989), patients taking antihypertensive agents in combination with azathioprine retained their RFR, whilst those taking CyA alone did not. McNally (unpublished observations) has also recently repeated this work in 10 normotensive patients taking CyA; again no RFR could be demonstrated. Thus it appears that CyA seems to inhibit or abolish RFR capacity in the transplanted kidney.

The mechanism by which protein stimulates RFR has been elucidated in some animal species but not fully in man. Glycine stimulates glucagon production, whilst either of these substances infused into an intact animal produces a rise in circulating levels of the glucuronide hormone known as Glomerulopressin (Uranga et al 1979, Alvestrand and Bergstrom 1984). This substance is produced in the liver and evidence suggests that it increases ERPF and GFR via prostaglandin mediators. This theory gains support from the fact that pre-treatment with a large dose of indomethacin inhibited the protein-induced stimulation of RFR (Eisenhauer et al 1985). However, in another study a smaller dose of NSAID did not produce the same effect and inadequate inhibition was suggested to explain this discrepancy (Herrera et al 1988).

Although Glomerulopressin has not been identified in man, it is interesting to note that poorly controlled diabetics who have hyperglucagonaemia demonstrate renal hyperfiltration (Parving et al 1977). In addition, cirrhotics do not demonstrate a RFR but in people
recovering from acute viral hepatitis this capacity returns once the condition has resolved (Dratwa et al 1987).

CyA is known to stimulate the renal production of the vasoconstrictor prostanoid TXA$_2$ (Jorkasky et al 1987) and may also inhibit the endothelial cell production of the vasodilatatory prostaglandin, prostacyclin (Brown and Neild 1987). Therefore CyA may abolish RFR by blocking production of its vasodilatatory prostanoid mediator, or by causing an imbalance of overall intra-renal prostanoid production in favour of vasoconstriction. Patients receiving azathioprine immunosuppression have no inhibition of intra-renal prostanoid production and consequently demonstrate a renal functional reserve.

Alternatively CyA is known to be hepatotoxic, although this is rarely seen at therapeutic doses in renal allograft recipients. However, doses used may be adequate to reduce the hepatic production of a Glomerulopressin-like substance necessary in the pathway which stimulates renal functional reserve.

It has been suggested that nifedipine could block RFR by inhibiting prostaglandin synthesis, however, patients taking azathioprine and nifedipine demonstrate no abolition of RFR. Calcium-channel blockade, therefore, does not appear to play an important role in the absence of RFR which is seen in all patients receiving CyA immunosuppression.

The majority of evidence to date indicates that treatment with CyA induces powerful intra-renal vasoconstriction. The effects of CyA upon endothelial, platelet and smooth muscle reactivity cannot be assumed to result solely from changes in prostanoid production, however, experimental work suggests that many factors are responsible for the effects seen, whilst the mediators described in animal models may differ
from those involved in man. The multiplicity of mechanisms incriminated is also supported by the fact that no single treatment has to date totally reversed the CyA-induced deterioration in renal function but several different approaches have produced a partial improvement.

Other inter-cellular chemicals have recently been implicated, two of which, Platelet Activating Factor (PAF) and Endothelium Derived Relaxing Factor (EDRF), have properties resembling those of TXA$_2$ and PGI$_2$ respectively. The synthesis of neither substance appears to be related to the arachidonic acid cascade but both may play a role in the pathogenesis of CyA nephrotoxicity.

PAF is a lipid released during immune responses by many cell types including renal cells and is involved in the inflammatory process. As its name implies, it stimulates platelets to aggregate and adhere to vascular endothelium. PAF alone may not have an effect on smooth muscle (Gresele 1989) but once platelets are activated, factors subsequently released or synthesised can induce vasoconstriction. In addition, in renal tissue culture, prostanoid synthesis is stimulated by PAF (Schlondorff and Neuwirth 1986). In the intact rat, infusion of PAF reduces renal blood flow, GFR and ultra-filtration coefficient, effects which are blocked by the co-administration of BN 52021, a PAF antagonist. A role for PAF in CyA nephrotoxicity has been demonstrated by the use of specific PAF antagonists (Pavao et al 1989). In a rat model of CyA nephrotoxicity induced by 50mg/kg/d, elevated urinary excretion of a PAF-like lipid was demonstrated. In addition, treatment with BN 52063, (a PAF antagonist) or verapamil, improved the nephrotoxic effects (Pirotzky et al 1988).

EDRF is a factor released by endothelial cells in response to mechanical
or chemical stimuli and has actions which closely parallel those of PGI₂. However, it differs in that its actions are characterised by a rise in intra-cellular cGMP concentration whilst PGI₂ stimulates cAMP synthesis. Both substances however, have a final common pathway reducing intra-cellular calcium availability (Moncada et al 1987). In 1987, Radomski et al reported that a substance released by endothelial cells in response to bradykinin stimulation, closely resembled the chemical nitric oxide (NO). This substance reduced platelet aggregation, relaxed vascular smooth muscle and had a much shorter half-life than PGI₂. In addition, no 6kPGF₁α could be detected in its presence. Experimental work has demonstrated nitric oxide to have identical properties to EDRF in vitro and most people now believe these to be one and the same substance. In contrast to PGI₂, EDRF is a much better inhibitor of platelet adhesion and its release may not rely on the movement of calcium ions (Pearson 1989). Overall, however, the activities of these two substances appear to be synergistic.

Despite this interesting work, strong clinical and experimental evidence confirms that CyA therapy greatly increases intra-renal synthesis of the vasoconstrictor prostanoid TXA₂. In addition, there is further evidence to suggest that this is compounded by decreased intra-renal synthesis of the vasodilatatory prostanoid PGI₂. An important role for these substances in the pathogenesis CyA nephrotoxicity is therefore proposed.

The large concentrations of TXB₂ excreted in the urine probably originate from local macrophages, monocytes and renal cells in addition to circulating platelets. Attempts to reduce the synthesis of TXA₂ using cyclo-oxygenase inhibitors were unsuccessful. Blockade of this enzyme system is non-selective, inhibiting the production of both vasoconstrictory and vasodilatatory prostaglandins.
Thromboxane synthase inhibition seemed a better approach but produced disappointing results. The drugs involved had only short durations of action which did not produce complete inhibition of the enzyme. High concentrations of precursor endoperoxides resulted, which were channelled into other synthetic pathways increasing the production of both PGD\(_2\) and PGE\(_2\) which have anti-platelet activities, in addition to providing a substrate for endothelial PGI\(_2\) production. PGF\(_{2\alpha}\) synthesis was also increased but has opposing effects of vasoconstriction. However, the excess endoperoxides produced within this system also inhibited the production of cAMP which plays an important role in vasodilatation. Any beneficial effect of increased production of vasodilatatory prostanoids tended to be antagonised by the thromboxane-mimetic effects of the endoperoxides PGG\(_2\) and PGH\(_2\).

Thromboxane receptor antagonism appeared to be the next logical step in attempting to ameliorate CyA nephrotoxicity as any additive effect of endoperoxide precursors would also be blocked.

Specific TXA\(_2\) receptor blockade was previously demonstrated during in vitro and ex vivo testing of GR32191B. Using a rat model of a renal isograft, which excluded any problems of rejection, Perico and co-workers (1990 in press) found the combination of this drug and CyA produced a significant improvement in SeCr and Cl\(_{\text{Cr}}\) measurements when compared with CyA given alone. However, renal function remained significantly worse than in animals who had received no CyA therapy. GR32191B had no effect upon the elevated urinary excretion of TXB\(_2\) induced by CyA treatment.

In another study, (Ahonen et al 1990) GR32191B was given to renal allograft recipients for the first 14 days after transplantation, on a
placebo-controlled basis. A problem was encountered with severe haematuria in two patients and pre-operative dosing was ceased. However, one year after transplantation, the treated group demonstrated significantly fewer early rejection episodes and improved graft survival, whilst SeCr concentrations were also lower in these patients.

In view of these results, an improvement in renal function was anticipated in the patients involved in the present study. However, neither of the studies reported included the concomitant use of a calcium-channel blocker.

Thus the renal allograft of a patient taking CyA is not working at maximum capacity. Further functional capacity exists and can be utilised if CyA therapy ceases. If CyA therapy is continued, a small improvement in function can be obtained by the addition of a calcium-channel blocker to the drug regimen. Theoretically, a similar effect should be produced in a patient not requiring antihypertensive therapy by adding a thromboxane receptor antagonist, as in Perico's rat model (et al 1990). To prove this theory, ideally, the effect of GR32191B should be investigated in patients not taking calcium-channel antagonists. However, if TXA$_2$ receptor blockade consistently reduces circulating 6kPGF$_{1a}$ concentrations, producing minimal if any improvement in CyA nephrotoxicity, there is no place for these drugs in the treatment of this condition.

Therefore, when considering other ways of improving renal function in patients who remain on CyA immunosuppression, modification of PGI$_2$ concentration is essential in addition to TXA$_2$ receptor antagonism. The combination of TXA$_2$ receptor blockade with TXA$_2$ synthase inhibition would channel excessive concentrations of endoperoxides into PGI$_2$. 

265
production (Gresele et al 1987), however, dissimilar pharmacokinetics make combination therapy difficult. Picotamide is an experimental drug which appears to have dual properties, suppressing stimulated platelet aggregation and favouring the endothelial synthesis of PGI$_2$ (Gresele et al 1989). There may be a future role for this agent in this field.

An alternative approach would be to give vasodilatatory prostanoids therapeutically in combination with CyA. Misoprostol is a synthetic PGE$_1$ analogue which produced fatal hypotension when given in large doses to rats. However, lower doses only incompletely reversed the nephrotoxicity induced by CyA (Paller 1988). Rats given PGE$_2$, the major dilating prostanoid in that species, demonstrated a nephrocytoprotective effect in addition to producing improvement in renal function (Makowka et al 1986, Pomer et al 1987). Ryfell et al (1986) demonstrated low trough concentrations and reduced immunosuppressive activity in these animals and suggested that PGE$_2$ acted by decreasing the absorption of CyA.

New trials using fish oil as the vehicle for CyA are about to commence. Fish oil contains eicosapentaenoic acid which participates in the prostaglandin synthetic pathway producing TXA$_3$ and PGI$_3$. TXA$_3$ is an inactive thromboxane and will therefore not induce vasoconstriction, whilst PGI$_3$ retains vasodilatatory and anti-aggregation activity. In a rat model of CyA nephrotoxicity, renal function was again partially improved by fish oil but prostacyclin (PGI$_2$ or PGI$_3$) production was still inhibited in the presence of CyA (Elzinga et al 1987a and b, Rogers et al 1988).

In conclusion, CyA induced nephrotoxicity is certainly multifactorial, altered prostaglandin synthesis being one important factor involved.
When attempting to modify the imbalance of intra-renal prostanoid production in the presence of CyA, maximum improvement in renal function is most likely to be achieved by the combination of TXA$_2$ receptor blockade and enhancement of circulating concentrations of vasodilatatory prostanoids.
APPENDICES
APPENDIX I

The measurement of:-

1. Serum creatinine
2. Urinary creatinine & creatinine clearance
3. Electrolytes, liver and bone function
4. Urinary protein (24 hour)
5. Haematological parameters
6. Urinalysis (dip stick)
7. Urinary electrolyte concentrations
8. Urinary glucose concentration
9. N-acetyl-beta-D-glucosaminidase (NAG)
All measurements of haematology, serum biochemistry and urinary creatinine were performed in the relevant laboratories at the Leicester General Hospital.

**BIOCHEMISTRY**

All serum measurements were made on a Technicon SMAC (Systematic Multiple channel Analyser with Computer) system (Technicon Instruments Corporation, Tarrytown, New York, USA). This machine can assay over 20 parameters from a single sample of serum. The results are assimilated by a computer and printed directly on to result forms. 100 samples can be analysed per hour in a system which employs continuous flow with bubble segmentation. Racks containing 8 samples each are inserted; the SMAC analyser re-calibrates after every 6th rack.

1. **SERUM CREATININE**

The chemical method utilised for quantification of creatinine is based on the colorimetric assay first described by Jaffe in 1886. When creatinine reacts with colourless picric acid in a solution rendered alkaline by the addition of sodium hydroxide, a red coloration is produced. The density of colour is measured by a spectrophotometer detecting light absorption at a wavelength of 505nm. Bonsnes and Taussky (1945) determined the ideal proportion of reagents for maximum colour production, whilst in 1961, Chasson and colleagues first described an automated form of this analysis.

The serum sample aspirated by the machine is diluted, then dialysed against a semi-permeable membrane to separate creatinine from protein and other substances which may interfere with the assay. Sodium hydroxide and the colour reagent containing picric acid are then added.
to the continuous flow. The resultant mixture is heated to 37°C, which accelerates development of the chromogen. The coloured solution passes through a flow cell and absorption at 505nm is measured.

Certain substances, described as "non-creatinine chromogens", can also produce red coloration in reaction with alkaline picrate. Their concentration depends on the method used for deproteinization and also the time allowed for colour development to occur. The list of interfering substances is long and includes: aceto-acetic acid, ascorbic acid, glucose, methyldopa, pyruvate, barbiturates and uric acid. In people with normal renal function, these substances can represent up to 15% of the chromogenic material in the analysis (Doolan et al 1962). The Jaffe reaction can therefore produce a serum creatinine measurement which is higher than true creatinine concentration. As serum creatinine levels rise with increasing renal dysfunction, this artefact becomes relatively less important. Thus the significance of non-creatinine chromogens is inversely proportional to actual serum creatinine levels (Bauer et al 1982).

When estimating creatinine clearance by the Jaffe reaction, in the presence of normal renal function, the over-estimation of serum creatinine (in P) is counterbalanced by the secreted element of urinary creatinine (in U). Therefore, when using the formula clearance = UV/P, creatinine clearance may more closely resemble true GFR (Renkin and Robinson 1984).

The laboratory quotes a calculated coefficient of variation of 5% for samples in the 100µmol/l range. At 500µmol/l, this is reduced to approximately 2%.
2 CREATININE CLEARANCE AND URINARY CREATININE

When a complete biochemical profile is required, the Technicon SMAC autoanalyser is used. When an urgent serum creatinine level is required, the Astra analyser (Beckman, High Wycombe, Bucks, UK.) is used. This analyser is also employed to measure serum and urinary creatinine concentrations required for the calculation of creatinine clearance.

The Astra analyser contains the Creatinine Chemistry Module. Again the Jaffe colorimetric reaction is utilised but the assay differs in some important details. Firstly, the complete reaction occurs within the reaction cell. This contains 2.6ml of alkaline picrate reagent and to it is added either 30μl of serum or 10μl of urine. The colour formation occurs as described, and the spectrophotometer measures light absorption at 520nm. The other important difference in this technique is that absorption is measured exactly 25.6 seconds after sample introduction. Modification of reagents has reduced some of the effects of "interfering substances", whilst kinetic studies have demonstrated that fast reacting pseudo-creatinines contribute to colour formation in the earlier part of the reaction. Rate of reaction appears to be proportional to creatinine concentration for a short time only, after which the slow reacting pseudo-creatinine substances contribute to total colour formation. Hence the critical time for measurement of absorption.

The Creatinine Chemistry Module produces results which are specific and available in minutes, very small samples are also suitable. The laboratory quotes a coefficient of variation of 5% for this method of determining creatinine concentration.
To calculate creatinine clearance:

\[
\text{24hr urine volume} \quad \frac{\text{V}}{1440} = \text{urine flow in ml/min.} = V
\]

urine creatinine = \( U \ \mu\text{mol/ml} \)

serum creatinine = \( P \ \mu\text{mol/l} \)

\[
C_{\text{Cr}} = \frac{U \times V}{P} \times 1000 \ \text{ml/min.}
\]

3 ELECTROLYTES, LIVER AND BONE FUNCTION

The sample can be analysed on the same day or serum separated for later analysis.

Sodium

The measurement of sodium concentration requires no chemical reaction and uses less than 15μl of serum. The buffered serum sample is heated to 55°C whilst flowing along the surface of a glass electrode. The sodium ions induce a measurable change in electrical potential.

Potassium

A similar potassium ion-selective electrode measures a proportional change in electrical potential when a buffered serum sample flow passes the membrane. Interference from sodium ions is insignificant with this electrode.

Total bilirubin

A colorimetric assay is used to measure bilirubin. Combination with a diazo-reagent forms a red azobilirubin complex. Strong alkali is added to standardise pH and dissolve proteins, producing a further colour change to blue alkaline azobilirubin. The final solution is a green colour resulting from mixing of all reagents plus endogenous pigment. A
blank channel contains the same reagents with the exception of the diazo-compound. Subtracted absorption produces the concentration of bilirubin alone.

**Gamma glutamyltransferase**
The capacity of this enzyme to catalyse the transfer of a gamma glutamyl group from a peptide to an L-amino acid after a short period of incubation is utilised. Subsequently the amount of peptide released is measured by light absorption.

**Alanine aminotransferase or glutamic-pyruvic transaminase**
Enzymic reaction is again utilised, the rate of reaction being measured by relative amounts of substrate:product at 3 timed-intervals. Concentration of GPT is measured by comparison of rate of reaction with that produced by known standard concentrations of enzyme.

**Alkaline phosphatase**
Alkaline phosphatase hydrolyses colourless p-nitrophenyl phosphate to yellow p-nitrophenol. After dialysis, to separate bilirubin which absorbs light at approximately the same wavelength as p-nitrophenol, the concentration of end-product is proportional to the concentration of enzyme in the sample.

**Calcium**
In a colorimetric reaction, Cresolphthalein Complexone binds calcium ions in an alkaline medium to produce a pink coloured complex. The serum sample is treated to remove interfering Mg\(^{++}\) ions and release protein bound Ca\(^{++}\) ions. The free calcium ions are then dialysed across a semi-permeable membrane into the complexing solution. The reaction temperature is maintained at 37°C.
**Albumin**

The measurement of albumin depends upon its combination with the dye bromcresol green (BCG). The serum sample is added to this dye whilst the pH of the mixture is regulated by a buffer within the BCG. This reaction occurs at room temperature, absorption is measured at 630nm.

---

**4 URINARY PROTEIN**

24-hour measurements of urinary protein were made in the Department of Biochemistry at the Leicester Royal Infirmary.

A turbidimetric method is used, employing a computerised COBAS BIO analyser (Roche, Welwyn, Herts, UK.). When urine rendered alkaline reacts with benzethonium chloride, a stable turbidity is produced independent of temperature. The urine sample to be analysed is first tested with a dipstick (Boehringer Mannheim). If the protein content is greater than the upper limit of the stick test, the urine sample is diluted as required with normal saline. The working range of the assay falls between 0.3 and 5.0g/l. Incubation is carried out for 10sec at 25°C, after which time two readings of optical density are made. The results obtained are compared with a standard curve and protein concentration is quoted in grams per litre. A simple calculation is performed to ascertain 24-hour urine protein losses from the volume of urine passed. Values below 0.3g/l are reported as "no excess present". The standard curve is produced from a range of internally produced Quality Control concentrations of protein diluted with saline and measured on the Technicon SMAC analyser.

A coefficient of variation of between 1.5 and 5% is quoted.

---

**5 HAEMATOLOGICAL PARAMETERS**

The haematology laboratory at Leicester General Hospital, uses a Coulter
Model S plus 4 counter (Coulter Electronics, Luton, England) for the automated measurement of samples.

Approximately 0.1ml of blood is aspirated from a fresh potassium EDTA-anticoagulated sample and divided into two aliquots. The first is subjected to a large dilution with an isotonic solution which maintains cellular integrity but enables counting of platelets and erythrocytes, both present in very large numbers. Counting is performed by means of a slit counter mechanism; cell size is also estimated at this stage.

The second aliquot is subjected to dilution with an hypertonic solution (Lyse S III Diff.), which lysed erythrocytes, converts haemoglobin to the CyanMet form, used for colorimetric analysis of haemoglobin concentration and also removes a thin layer of leukocyte cytoplasm. CyanMet haemoglobin absorbs light at 420nm and the concentration in solution is calculated against a panel of blanks and standard concentrations.

From this same second aliquot of blood the leukocyte count is performed with a slit counting system which also grades the cells on the basis of their size. A graphical representation of white cell size is produced, normally demonstrating three peaks (lymphocytes between 35 and 90 \( \mu m^3 \), mononuclear cells between 90 and 160\( \mu m^3 \) and granulocytes between 160 and 450\( \mu m^3 \)). Any abnormalities are confirmed by a manual stained film.

6 URINALYSIS (DIP STICK)

On each visit to the hospital, a fresh specimen of urine was tested with a BM test 5-L urinalysis stick (Boehringer Corporation [London] Ltd., Bell Lane, Lewes, East Sussex), for the presence of blood and protein. The specific reagent is contained in a layer of absorbent paper covered
by a nylon mesh which allows rapid and uniform penetration of urine. All reactions are colorimetric and are interpreted by comparison with a colour chart, after a 60-second incubation period.

i) Protein

Tetra-chlorophenol tetra-bromosulphthalein changes colour from yellow to light green in the presence of protein at constant pH. The strip is sensitive to 0.06g/l of albumin, well below the physiologically accepted limit of 0.3g/l in the first morning specimen.

+ = 0.3g/l of protein
++ = 1.0g/l
+++ = 5g/l or more.

ii) Blood

Both haemoglobin and myoglobin catalyse the oxidation of the indicator on the reagent strip causing it to change colour from yellow to blue-green. Scattered or compacted green dots indicate intact erythrocytes which have been haemolysed, their haemoglobin being released locally. The test is sensitive to 5RBC /μl of urine. More than this number is considered pathological.

+ = denotes 5-15 RBC/μl
++ = 30 and 100 RBC/μl
+++ = 150 -300 RBC/μl.

The following measurements were performed in the laboratories of the Department of Chemical Pathology, Aberdeen Royal Infirmary, Scotland. All measurements were made on an aliquot taken from a 24-hour collection of urine performed on the day prior to isotope estimations of renal function. The aliquots were stored frozen at -20°C until the assays could be performed. 24-hour concentration was calculated from the total
volume of urine produced.

7 URINARY ELECTROLYTE CONCENTRATIONS

Measurements of 24-hour excretion of both sodium and potassium were made using an IL 943 flame emission spectrometer, (Instrumentation Laboratories Ltd., Warrington, UK).

8 URINARY GLUCOSE CONCENTRATIONS

Glucose was measured using a Beckman Glucose Analyser, (Beckman Instruments Inc., Fullerton, CA, USA.). When glucose in the sample is converted to gluconate by glucose oxidase reagent, oxygen consumption occurs. One molecule of \( O_2 \) is consumed for every molecule of glucose converted. The partial pressure of oxygen in the solution is measured with an oxygen electrode, loss of oxygen being directly proportional to the concentration of glucose within the sample.

9 N-ACETYL-beta-D-GLUCOSAMINIDASE (NAG)

N-acetyl-beta-D-glucosaminidase is a lysosomal enzyme which participates in the degradation of mucopolysaccharides and glycoproteins and is found in renal proximal tubular cells (Whiting et al 1979). The presence of large concentrations of this substance in urine is thought to indicate renal tubular damage and is demonstrated in renal transplant rejection and also in diabetes mellitus.

Urinary concentrations can be measured from a single specimen, however, results more suitable for comparison are obtained from 24-hour collections. NAG is expressed in units per mg of creatinine, which compensates for variations in NAG concentration which may result from concentrated or diluted urine samples.

The assay measures enzyme activity within a sample in the presence of a
suitable substrate.

0.1ml of urine is added to 0.9ml of a citrate-phosphate buffer which maintains a pH of 4.3. This solution is equilibrated at 37°C for 5 minutes.

1.0ml of substrate (2mmol of 4 methyl-umbelliferyl-2-acetoamido-2-deoxy-β-D-glucopyranoside) is added and the mixture incubated for 30 minutes. The reaction releases 4 methylumbelliferone (4MU) which is quantitated fluorimetrically using an excitation wavelength of 360nm; detecting at 440nm. Standards and blanks, containing enzyme or substrate, are included. The reaction is terminated with 3.0ml of 0.5M glycine sodium hydroxide buffer with a pH of 10.4.

Assay of known concentrations of NAG has demonstrated a linear release of 4MU up to 45 minutes, to a maximum capacity of 3000nmol of 4MU per hour per ml of urine.

1 unit of enzyme activity is defined as 1mmol of 4MU released per hour at 37°C.
# APPENDIX II

## MEDIAN VALUES OF FUNCTION FOR PLACEBO-TREATED PATIENTS OVER THE 12-MONTH PERIOD OF INVESTIGATION

<table>
<thead>
<tr>
<th>Tx</th>
<th>onset</th>
<th>6/12</th>
<th>12/12</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1986</td>
<td>55.9</td>
<td>56</td>
<td>50.9</td>
</tr>
<tr>
<td>1987</td>
<td>40.2</td>
<td>36.8</td>
<td>37.5</td>
</tr>
<tr>
<td>1988</td>
<td>43.8</td>
<td>36.6</td>
<td>30.8</td>
</tr>
<tr>
<td>ERPF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1986</td>
<td>300.5</td>
<td>262</td>
<td>256.3</td>
</tr>
<tr>
<td>1987</td>
<td>239</td>
<td>179</td>
<td>187.4</td>
</tr>
<tr>
<td>1988</td>
<td>199.5</td>
<td>204</td>
<td>160.3</td>
</tr>
<tr>
<td>ClCr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1986</td>
<td>67.6</td>
<td>52.3</td>
<td>67.7</td>
</tr>
<tr>
<td>1987</td>
<td>62.9</td>
<td>41.0</td>
<td>42.9</td>
</tr>
<tr>
<td>1988</td>
<td>53.7</td>
<td>40.5</td>
<td>31</td>
</tr>
<tr>
<td>SeCr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1986</td>
<td>116.5</td>
<td>120.5</td>
<td>123</td>
</tr>
<tr>
<td>1987</td>
<td>190</td>
<td>192.5</td>
<td>196</td>
</tr>
<tr>
<td>1988</td>
<td>157</td>
<td>152</td>
<td>239</td>
</tr>
<tr>
<td>RVR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1986</td>
<td>31.3</td>
<td>40.9</td>
<td>40.3</td>
</tr>
<tr>
<td>1987</td>
<td>41.3</td>
<td>63.4</td>
<td>49.5</td>
</tr>
<tr>
<td>1988</td>
<td>57.1</td>
<td>56.3</td>
<td>75.9</td>
</tr>
<tr>
<td>CyA dose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1986</td>
<td>6.75</td>
<td>6.75</td>
<td>6</td>
</tr>
<tr>
<td>1987</td>
<td>5.75</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>1988</td>
<td>7</td>
<td>6</td>
<td>4.5</td>
</tr>
<tr>
<td>CyA level</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1986</td>
<td>332</td>
<td>203</td>
<td>251</td>
</tr>
<tr>
<td>1987</td>
<td>372</td>
<td>197.5</td>
<td>264</td>
</tr>
<tr>
<td>1988</td>
<td>298</td>
<td>227</td>
<td>212</td>
</tr>
</tbody>
</table>

GFR, ERPF, ClCr: ml/min  
SeCr: µmol/l  
CyA dose: mg/kg/day  
CyA level: ng/ml  
RVR: dyne sec/cm²
### RAW DATA FROM PATIENTS INVESTIGATED

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFR</td>
<td>ml/min</td>
</tr>
<tr>
<td>SeCr</td>
<td>μmol/l</td>
</tr>
<tr>
<td>ERPF</td>
<td>ml/min</td>
</tr>
<tr>
<td>Cl\textsubscript{Cr}</td>
<td>ml/min</td>
</tr>
<tr>
<td>BP</td>
<td>mmHg</td>
</tr>
<tr>
<td>RVR</td>
<td>dyne sec/cm\textsuperscript{5}</td>
</tr>
<tr>
<td>CyA dose</td>
<td>mg/kg/d</td>
</tr>
<tr>
<td>CyA level</td>
<td>ng/ml</td>
</tr>
</tbody>
</table>
### APPENDIX III

#### Placebo group before treatment

<table>
<thead>
<tr>
<th>CODE</th>
<th>1st GFR</th>
<th>1st SeCr</th>
<th>1st ERPF</th>
<th>1st ClCr</th>
<th>1st BP</th>
<th>1st RVR</th>
<th>1st CyA dose</th>
<th>1st CyA level</th>
</tr>
</thead>
<tbody>
<tr>
<td>102</td>
<td>57.1</td>
<td>157</td>
<td>151</td>
<td>66.8</td>
<td>135/80</td>
<td>65.12</td>
<td>7.0</td>
<td>260</td>
</tr>
<tr>
<td>104</td>
<td>34.6</td>
<td>158</td>
<td>210</td>
<td>40.1</td>
<td>160/100</td>
<td>57.14</td>
<td>5.0</td>
<td>273</td>
</tr>
<tr>
<td>105</td>
<td>40</td>
<td>106</td>
<td>199.5</td>
<td>88.2</td>
<td>140/80</td>
<td>50.13</td>
<td>4.0</td>
<td>298</td>
</tr>
<tr>
<td>108</td>
<td>22.5</td>
<td>242</td>
<td>109</td>
<td>34.4</td>
<td>135/80</td>
<td>90.21</td>
<td>3.0</td>
<td>89</td>
</tr>
<tr>
<td>109</td>
<td>23.2</td>
<td>306</td>
<td>152</td>
<td>24.4</td>
<td>120/80</td>
<td>61.24</td>
<td>9.6</td>
<td>763</td>
</tr>
<tr>
<td>111</td>
<td>43.8</td>
<td>192</td>
<td>160</td>
<td></td>
<td>140/80</td>
<td>62.5</td>
<td>8.0</td>
<td>186</td>
</tr>
<tr>
<td>113</td>
<td>58.6</td>
<td>124</td>
<td>309</td>
<td>49</td>
<td>125/85</td>
<td>31.82</td>
<td>6.0</td>
<td>685</td>
</tr>
<tr>
<td>114</td>
<td>47.8</td>
<td>120</td>
<td>317</td>
<td>69</td>
<td>130/90</td>
<td>32.6</td>
<td>12.0</td>
<td>305</td>
</tr>
<tr>
<td>117</td>
<td>46.4</td>
<td>128</td>
<td>361</td>
<td>58.4</td>
<td>100/70</td>
<td>22.16</td>
<td>7.0</td>
<td>445</td>
</tr>
<tr>
<td>201</td>
<td>39.9</td>
<td>229</td>
<td>156</td>
<td>32.3</td>
<td>120/80</td>
<td>59.83</td>
<td>10.0</td>
<td>489</td>
</tr>
<tr>
<td>203</td>
<td>57.4</td>
<td>100</td>
<td>286</td>
<td>67.5</td>
<td>125/80</td>
<td>33.22</td>
<td>6.0</td>
<td>439</td>
</tr>
<tr>
<td>206</td>
<td>28.6</td>
<td>271</td>
<td>112</td>
<td>24.7</td>
<td>190/90</td>
<td>110.12</td>
<td>4.0</td>
<td>196</td>
</tr>
<tr>
<td>207</td>
<td>61.7</td>
<td>133</td>
<td>420</td>
<td>78</td>
<td>100/60</td>
<td>17.46</td>
<td>7.5</td>
<td>410</td>
</tr>
<tr>
<td>303</td>
<td>40.5</td>
<td>154</td>
<td>205</td>
<td>117</td>
<td>120/70</td>
<td>42.28</td>
<td>5.5</td>
<td>334</td>
</tr>
<tr>
<td>304</td>
<td>38</td>
<td>226</td>
<td>273</td>
<td>58.2</td>
<td>150/90</td>
<td>40.29</td>
<td>4.5</td>
<td>329</td>
</tr>
<tr>
<td>401</td>
<td>40.5</td>
<td>115</td>
<td>127</td>
<td>56.7</td>
<td>130/90</td>
<td>81.36</td>
<td>5.0</td>
<td>351</td>
</tr>
<tr>
<td>402</td>
<td>63</td>
<td>118</td>
<td>385</td>
<td>63</td>
<td>140/90</td>
<td>27.71</td>
<td>6.5</td>
<td>241</td>
</tr>
<tr>
<td>403</td>
<td>57</td>
<td>76</td>
<td>375</td>
<td>59.2</td>
<td>140/90</td>
<td>28.44</td>
<td>5.0</td>
<td>193</td>
</tr>
<tr>
<td>405</td>
<td>28</td>
<td>249</td>
<td>130</td>
<td>79</td>
<td>170/110</td>
<td>100</td>
<td>10.0</td>
<td>269</td>
</tr>
<tr>
<td>410</td>
<td>47.3</td>
<td>126</td>
<td>162</td>
<td>66.5</td>
<td>160/90</td>
<td>69.96</td>
<td>4.5</td>
<td>1263</td>
</tr>
<tr>
<td>412</td>
<td>62.9</td>
<td>100</td>
<td>283</td>
<td>82.2</td>
<td>120/85</td>
<td>34.16</td>
<td>9.0</td>
<td>582</td>
</tr>
<tr>
<td>414</td>
<td>55.3</td>
<td>121</td>
<td>318</td>
<td>68.6</td>
<td>130/70</td>
<td>28.3</td>
<td>7.3</td>
<td>334</td>
</tr>
<tr>
<td>416</td>
<td>56.5</td>
<td>98</td>
<td>379</td>
<td>89.4</td>
<td>90/60</td>
<td>18.47</td>
<td>7.0</td>
<td>330</td>
</tr>
</tbody>
</table>
### Placebo group after 3 months' of treatment

<table>
<thead>
<tr>
<th>CODE</th>
<th>1st GFR</th>
<th>1st Scr</th>
<th>1st ERPF</th>
<th>1st ClCr</th>
<th>1st BP</th>
<th>1st RVR</th>
<th>1st CyA dose</th>
<th>1st CyA level</th>
</tr>
</thead>
<tbody>
<tr>
<td>102</td>
<td>49</td>
<td>137</td>
<td>204</td>
<td>109</td>
<td>145/80</td>
<td>49.84</td>
<td>7.0</td>
<td>586</td>
</tr>
<tr>
<td>104</td>
<td>25</td>
<td>190</td>
<td>185.5</td>
<td>173</td>
<td>140/80</td>
<td>53.91</td>
<td>4.5</td>
<td>246</td>
</tr>
<tr>
<td>105</td>
<td>33.8</td>
<td>115</td>
<td>109</td>
<td>38.8</td>
<td>130/70</td>
<td>82.57</td>
<td>4.0</td>
<td>335</td>
</tr>
<tr>
<td>108</td>
<td>33.6</td>
<td>238</td>
<td>129</td>
<td>36.2</td>
<td>130/80</td>
<td>74.94</td>
<td>3.0</td>
<td>103</td>
</tr>
<tr>
<td>109</td>
<td>28</td>
<td>261</td>
<td>166</td>
<td>35</td>
<td>140/90</td>
<td>64.26</td>
<td>6.0</td>
<td>114</td>
</tr>
<tr>
<td>111</td>
<td>34.5</td>
<td>196</td>
<td>168.9</td>
<td>34</td>
<td>150/90</td>
<td>65.13</td>
<td>8.0</td>
<td>244</td>
</tr>
<tr>
<td>113</td>
<td>68.4</td>
<td>114</td>
<td>435</td>
<td>60</td>
<td>120/80</td>
<td>21.46</td>
<td>6.0</td>
<td>576</td>
</tr>
<tr>
<td>114</td>
<td>55.1</td>
<td>107</td>
<td>272</td>
<td>77.7</td>
<td>110/70</td>
<td>30.64</td>
<td>6.0</td>
<td>227</td>
</tr>
<tr>
<td>117</td>
<td>55.4</td>
<td>107</td>
<td>326.5</td>
<td>43.5</td>
<td>100/70</td>
<td>24.5</td>
<td>6.0</td>
<td>287</td>
</tr>
<tr>
<td>201</td>
<td>30.9</td>
<td>208</td>
<td>162</td>
<td>29.6</td>
<td>120/90</td>
<td>61.73</td>
<td>10.0</td>
<td>542</td>
</tr>
<tr>
<td>203</td>
<td>59.6</td>
<td>99</td>
<td>327</td>
<td>74.1</td>
<td>130/70</td>
<td>27.52</td>
<td>6.0</td>
<td>351</td>
</tr>
<tr>
<td>206</td>
<td>25</td>
<td>253</td>
<td>139</td>
<td>15</td>
<td>150/80</td>
<td>74.34</td>
<td>4.0</td>
<td>185</td>
</tr>
<tr>
<td>207</td>
<td>70.3</td>
<td>105</td>
<td>243</td>
<td>103.9</td>
<td>115/60</td>
<td>32.24</td>
<td>7.5</td>
<td>514</td>
</tr>
<tr>
<td>303</td>
<td>50.3</td>
<td>145</td>
<td>236</td>
<td>55.6</td>
<td>130/75</td>
<td>39.55</td>
<td>6.0</td>
<td>292</td>
</tr>
<tr>
<td>304</td>
<td>32.1</td>
<td>214</td>
<td>235</td>
<td>56.7</td>
<td>130/80</td>
<td>41.13</td>
<td>4.5</td>
<td>956</td>
</tr>
<tr>
<td>401</td>
<td>47</td>
<td>112</td>
<td>183</td>
<td>60.1</td>
<td>130/90</td>
<td>56.47</td>
<td>5.0</td>
<td>579</td>
</tr>
<tr>
<td>402</td>
<td>71.8</td>
<td>111</td>
<td>471.2</td>
<td>78.4</td>
<td>120/80</td>
<td>19.81</td>
<td>6.5</td>
<td>203</td>
</tr>
<tr>
<td>403</td>
<td>84.3</td>
<td>74</td>
<td>469</td>
<td>44</td>
<td>120/80</td>
<td>19.9</td>
<td>5.0</td>
<td>352</td>
</tr>
<tr>
<td>405</td>
<td>25.6</td>
<td>295</td>
<td>127</td>
<td>22.4</td>
<td>140/90</td>
<td>83.99</td>
<td>10.0</td>
<td>272</td>
</tr>
<tr>
<td>410</td>
<td>41.7</td>
<td>117</td>
<td>232</td>
<td>105</td>
<td>170/100</td>
<td>53.16</td>
<td>4.5</td>
<td>184</td>
</tr>
<tr>
<td>412</td>
<td>64.6</td>
<td>91</td>
<td>246</td>
<td>75.3</td>
<td>110/80</td>
<td>36.59</td>
<td>9.5</td>
<td>376</td>
</tr>
<tr>
<td>414</td>
<td>45.9</td>
<td>123</td>
<td>233</td>
<td>72</td>
<td>130/80</td>
<td>41.49</td>
<td>8.0</td>
<td>239</td>
</tr>
<tr>
<td>416</td>
<td>65.3</td>
<td>109</td>
<td>267.9</td>
<td>65.1</td>
<td>110/70</td>
<td>31.11</td>
<td>7.0</td>
<td>163</td>
</tr>
</tbody>
</table>
Placebo group at 6 months

<table>
<thead>
<tr>
<th>CODE</th>
<th>1st GFR</th>
<th>1st SeCr</th>
<th>1st ERPF</th>
<th>1st ClCr</th>
<th>1st BP</th>
<th>1st RVR</th>
<th>1st CyA dose</th>
<th>1st CyA level</th>
</tr>
</thead>
<tbody>
<tr>
<td>102</td>
<td>37.8</td>
<td>152</td>
<td>170</td>
<td>73.5</td>
<td>140/80</td>
<td>58.82</td>
<td>7.0</td>
<td>272</td>
</tr>
<tr>
<td>104</td>
<td>24</td>
<td>191</td>
<td>207</td>
<td>43</td>
<td>150/100</td>
<td>56.33</td>
<td>4.5</td>
<td>317</td>
</tr>
<tr>
<td>105</td>
<td>29.2</td>
<td>115</td>
<td>153</td>
<td>30.6</td>
<td>150/90</td>
<td>71.9</td>
<td>4.0</td>
<td>407</td>
</tr>
<tr>
<td>106</td>
<td>36.6</td>
<td>234</td>
<td>161</td>
<td>32</td>
<td>140/80</td>
<td>62.11</td>
<td>3.0</td>
<td>104</td>
</tr>
<tr>
<td>109</td>
<td>34.8</td>
<td>234</td>
<td>174</td>
<td>40.5</td>
<td>160/100</td>
<td>68.97</td>
<td>5.0</td>
<td>205</td>
</tr>
<tr>
<td>111</td>
<td>30.8</td>
<td>208</td>
<td>204</td>
<td>36.5</td>
<td>160/90</td>
<td>55.56</td>
<td>8.0</td>
<td>227</td>
</tr>
<tr>
<td>113</td>
<td>69</td>
<td>142</td>
<td>358.6</td>
<td>35.3</td>
<td>150/100</td>
<td>32.53</td>
<td>6.0</td>
<td>422</td>
</tr>
<tr>
<td>114</td>
<td>48.7</td>
<td>137</td>
<td>263.6</td>
<td>53.2</td>
<td>140/90</td>
<td>40.47</td>
<td>6.0</td>
<td>178</td>
</tr>
<tr>
<td>117</td>
<td>68.4</td>
<td>113</td>
<td>355</td>
<td>50</td>
<td>110/70</td>
<td>23.47</td>
<td>6.0</td>
<td>215</td>
</tr>
<tr>
<td>201</td>
<td>33.4</td>
<td>266</td>
<td>154</td>
<td>35.4</td>
<td>150/100</td>
<td>75.76</td>
<td>10.00</td>
<td>219</td>
</tr>
<tr>
<td>203</td>
<td>57.9</td>
<td>95</td>
<td>288</td>
<td>77</td>
<td>130/80</td>
<td>33.56</td>
<td>6.0</td>
<td>293</td>
</tr>
<tr>
<td>206</td>
<td>20.6</td>
<td>261</td>
<td>119</td>
<td>25</td>
<td>160/90</td>
<td>95.24</td>
<td>4.0</td>
<td>176</td>
</tr>
<tr>
<td>207</td>
<td>55</td>
<td>137</td>
<td>308</td>
<td>76</td>
<td>160/100</td>
<td>38.96</td>
<td>7.5</td>
<td>310</td>
</tr>
<tr>
<td>303</td>
<td>40.1</td>
<td>155</td>
<td>157</td>
<td>46.7</td>
<td>140/80</td>
<td>63.69</td>
<td>6.0</td>
<td>95</td>
</tr>
<tr>
<td>304</td>
<td>27</td>
<td>230</td>
<td>201</td>
<td>27</td>
<td>160/110</td>
<td>65.02</td>
<td>4.5</td>
<td>109</td>
</tr>
<tr>
<td>401</td>
<td>37.7</td>
<td>118</td>
<td>211</td>
<td>46</td>
<td>120/80</td>
<td>44.23</td>
<td>5.0</td>
<td>221</td>
</tr>
<tr>
<td>402</td>
<td>89.5</td>
<td>123</td>
<td>386</td>
<td>73</td>
<td>140/105</td>
<td>30.22</td>
<td>6.5</td>
<td>140</td>
</tr>
<tr>
<td>403</td>
<td>63.4</td>
<td>113</td>
<td>270</td>
<td></td>
<td>150/100</td>
<td>43.21</td>
<td>5.0</td>
<td>197</td>
</tr>
<tr>
<td>405</td>
<td>20.8</td>
<td>279</td>
<td>57.4</td>
<td>21</td>
<td>190/100</td>
<td>226.48</td>
<td>10.00</td>
<td>177</td>
</tr>
<tr>
<td>410</td>
<td>35.7</td>
<td>144</td>
<td>129</td>
<td>52.3</td>
<td>150/100</td>
<td>90.44</td>
<td>4.5</td>
<td>205</td>
</tr>
<tr>
<td>412</td>
<td>63.1</td>
<td>92</td>
<td>258</td>
<td>72.3</td>
<td>120/90</td>
<td>38.76</td>
<td>9.5</td>
<td>203</td>
</tr>
<tr>
<td>414</td>
<td>59</td>
<td>123</td>
<td>266.9</td>
<td>70.5</td>
<td>115/75</td>
<td>33.1</td>
<td>8.0</td>
<td>502</td>
</tr>
<tr>
<td>416</td>
<td>53</td>
<td>109</td>
<td>308</td>
<td>51.2</td>
<td>110/70</td>
<td>27.06</td>
<td>7.0</td>
<td>203</td>
</tr>
</tbody>
</table>
### Placebo group at 12 months

<table>
<thead>
<tr>
<th>CODE</th>
<th>1st GFR</th>
<th>1st SeCr</th>
<th>1st ERPF</th>
<th>1st ClCr</th>
<th>1st BP</th>
<th>1st RVR</th>
<th>1st CyA dose</th>
<th>1st CyA level</th>
</tr>
</thead>
<tbody>
<tr>
<td>102</td>
<td>58.9</td>
<td>146</td>
<td>237</td>
<td>77.6</td>
<td>130/90</td>
<td>43.6</td>
<td>7.0</td>
<td>212</td>
</tr>
<tr>
<td>104</td>
<td>19.4</td>
<td>256</td>
<td>141.5</td>
<td>29.1</td>
<td>160/100</td>
<td>84.81</td>
<td>5.0</td>
<td>286</td>
</tr>
<tr>
<td>105</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>108</td>
<td>24.1</td>
<td>371</td>
<td>110.2</td>
<td>22</td>
<td>135/80</td>
<td>89.23</td>
<td>3.0</td>
<td>0</td>
</tr>
<tr>
<td>109</td>
<td>37.4</td>
<td>222</td>
<td>179</td>
<td>32.9</td>
<td>160/100</td>
<td>67</td>
<td>4.0</td>
<td>215</td>
</tr>
<tr>
<td>111</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>113</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>114</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>117</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>201</td>
<td>39.1</td>
<td>232</td>
<td>161.2</td>
<td>27</td>
<td>130/80</td>
<td>91</td>
<td>8.0</td>
<td>240</td>
</tr>
<tr>
<td>203</td>
<td>69</td>
<td>98</td>
<td>333</td>
<td>85</td>
<td>120/85</td>
<td>29.03</td>
<td>6.0</td>
<td>288</td>
</tr>
<tr>
<td>206</td>
<td>16.8</td>
<td>258</td>
<td>103.7</td>
<td>22.5</td>
<td>140/90</td>
<td>102.86</td>
<td>4.0</td>
<td>211</td>
</tr>
<tr>
<td>207</td>
<td>57.9</td>
<td>131</td>
<td>315.5</td>
<td>62.9</td>
<td>160/80</td>
<td>33.81</td>
<td>7.5</td>
<td>340</td>
</tr>
<tr>
<td>303</td>
<td>35.9</td>
<td>160</td>
<td>195</td>
<td>54</td>
<td>130/80</td>
<td>49.57</td>
<td>6.0</td>
<td>314</td>
</tr>
<tr>
<td>304</td>
<td>32.3</td>
<td>283</td>
<td>179</td>
<td>31.7</td>
<td>150/90</td>
<td>61.18</td>
<td>4.5</td>
<td>236</td>
</tr>
<tr>
<td>401</td>
<td>50.9</td>
<td>98</td>
<td>217.1</td>
<td>62.7</td>
<td>115/65</td>
<td>37.62</td>
<td>5.0</td>
<td>377</td>
</tr>
<tr>
<td>402</td>
<td>80.5</td>
<td>112</td>
<td>388</td>
<td>80</td>
<td>150/90</td>
<td>28.35</td>
<td>6.5</td>
<td>247</td>
</tr>
<tr>
<td>403</td>
<td>68.4</td>
<td>123</td>
<td>279.1</td>
<td>62.4</td>
<td>140/100</td>
<td>40.61</td>
<td>5.0</td>
<td>255</td>
</tr>
<tr>
<td>405</td>
<td>20.4</td>
<td>318</td>
<td>116</td>
<td>13.8</td>
<td>160/100</td>
<td>103.45</td>
<td>6.0</td>
<td>244</td>
</tr>
<tr>
<td>410</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>412</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>414</td>
<td>50</td>
<td>128</td>
<td>256.3</td>
<td>67</td>
<td>130/90</td>
<td>40.32</td>
<td>8.0</td>
<td>411</td>
</tr>
<tr>
<td>416</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## APPENDIX IV

### Drug group before treatment with GR32191B

<table>
<thead>
<tr>
<th>CODE</th>
<th>1st GFR</th>
<th>1st SeCr</th>
<th>1st ERPF</th>
<th>1st ClCr</th>
<th>1st BP</th>
<th>1st RVR</th>
<th>1st CyA dose</th>
<th>1st CyA level</th>
</tr>
</thead>
<tbody>
<tr>
<td>101</td>
<td>38.3</td>
<td>116</td>
<td>199</td>
<td>44.2</td>
<td>160/100</td>
<td>60.3</td>
<td>4.5</td>
<td>449</td>
</tr>
<tr>
<td>103</td>
<td>43</td>
<td>107</td>
<td>193</td>
<td>118</td>
<td>170/110</td>
<td>67.36</td>
<td>6.0</td>
<td>208</td>
</tr>
<tr>
<td>106</td>
<td>30.6</td>
<td>150</td>
<td>250</td>
<td>41.6</td>
<td>130/90</td>
<td>41.33</td>
<td>9.5</td>
<td>230</td>
</tr>
<tr>
<td>107</td>
<td>51.2</td>
<td>134</td>
<td>301</td>
<td>52.9</td>
<td>130/70</td>
<td>29.9</td>
<td>10.0</td>
<td>192</td>
</tr>
<tr>
<td>112</td>
<td>36.6</td>
<td>120</td>
<td>225</td>
<td>49</td>
<td>140/90</td>
<td>47.41</td>
<td>9.8</td>
<td>612</td>
</tr>
<tr>
<td>115</td>
<td>55.9</td>
<td>109</td>
<td>313</td>
<td>53</td>
<td>100/65</td>
<td>24.49</td>
<td>6.0</td>
<td>314</td>
</tr>
<tr>
<td>118</td>
<td>64.2</td>
<td>92</td>
<td>414</td>
<td>68</td>
<td>100/60</td>
<td>17.71</td>
<td>6.0</td>
<td>275</td>
</tr>
<tr>
<td>202</td>
<td>34.1</td>
<td>191</td>
<td>189</td>
<td>24.7</td>
<td>110/50</td>
<td>37.04</td>
<td>6.0</td>
<td>437</td>
</tr>
<tr>
<td>204</td>
<td>42.7</td>
<td>221</td>
<td>255</td>
<td>44.2</td>
<td>140/80</td>
<td>39.22</td>
<td>7.0</td>
<td>336</td>
</tr>
<tr>
<td>301</td>
<td>40.3</td>
<td>177</td>
<td>267</td>
<td>120/90</td>
<td>37.45</td>
<td>7.0</td>
<td>328</td>
<td></td>
</tr>
<tr>
<td>302</td>
<td>73.9</td>
<td>111</td>
<td>260</td>
<td>107</td>
<td>160/100</td>
<td>46.15</td>
<td>6.5</td>
<td>254</td>
</tr>
<tr>
<td>305</td>
<td>36.2</td>
<td>262</td>
<td>154</td>
<td>31</td>
<td>160/90</td>
<td>73.59</td>
<td>4.0</td>
<td>307</td>
</tr>
<tr>
<td>306</td>
<td>56.3</td>
<td>136</td>
<td>213</td>
<td>59.5</td>
<td>150/90</td>
<td>51.64</td>
<td>4.0</td>
<td>209</td>
</tr>
<tr>
<td>404</td>
<td>34.6</td>
<td>181</td>
<td>186</td>
<td>36.3</td>
<td>120/70</td>
<td>46.59</td>
<td>4.5</td>
<td>228</td>
</tr>
<tr>
<td>406</td>
<td>50.2</td>
<td>131</td>
<td>265</td>
<td>77.3</td>
<td>130/100</td>
<td>41.51</td>
<td>6.5</td>
<td>196</td>
</tr>
<tr>
<td>407</td>
<td>55.3</td>
<td>122</td>
<td>306</td>
<td>44.6</td>
<td>140/100</td>
<td>37.04</td>
<td>6.5</td>
<td>273</td>
</tr>
<tr>
<td>408</td>
<td>27.6</td>
<td>226</td>
<td>204.2</td>
<td>32.3</td>
<td>180/90</td>
<td>58.77</td>
<td>6.5</td>
<td>301</td>
</tr>
<tr>
<td>409</td>
<td>48.9</td>
<td>111</td>
<td>270.9</td>
<td>47.3</td>
<td>140/100</td>
<td>41.84</td>
<td>4.5</td>
<td>484</td>
</tr>
<tr>
<td>411</td>
<td>41.3</td>
<td>151</td>
<td>136</td>
<td>58.5</td>
<td>160/80</td>
<td>78.43</td>
<td>4.0</td>
<td>325</td>
</tr>
<tr>
<td>413</td>
<td>63</td>
<td>113</td>
<td>218</td>
<td>90.1</td>
<td>118/70</td>
<td>39.45</td>
<td>5.5</td>
<td>246</td>
</tr>
<tr>
<td>415</td>
<td>42.1</td>
<td>125</td>
<td>186.3</td>
<td>62</td>
<td>140/90</td>
<td>57.26</td>
<td>4.0</td>
<td>198</td>
</tr>
</tbody>
</table>
### Drug group after 3 months' treatment with GR32191B

<table>
<thead>
<tr>
<th>CODE</th>
<th>1st GFR</th>
<th>1st SeCr</th>
<th>1st ERPF</th>
<th>1st Cl(_{Cr})</th>
<th>1st BP</th>
<th>1st RVR</th>
<th>1st CyA dose</th>
<th>1st CyA level</th>
</tr>
</thead>
<tbody>
<tr>
<td>101</td>
<td>32.4</td>
<td>141</td>
<td>152</td>
<td>58</td>
<td>140/70</td>
<td>61.4</td>
<td>4.0</td>
<td>460</td>
</tr>
<tr>
<td>103</td>
<td>47.5</td>
<td>101</td>
<td>187</td>
<td>52</td>
<td>140/90</td>
<td>57.04</td>
<td>6.0</td>
<td>333</td>
</tr>
<tr>
<td>106</td>
<td>31.3</td>
<td>191</td>
<td>208</td>
<td>36.3</td>
<td>110/90</td>
<td>46.47</td>
<td>6.5</td>
<td>85</td>
</tr>
<tr>
<td>107</td>
<td>24.3</td>
<td>173</td>
<td>180</td>
<td>65</td>
<td>140/90</td>
<td>59.26</td>
<td>9.0</td>
<td>308</td>
</tr>
<tr>
<td>112</td>
<td>43.9</td>
<td>124</td>
<td>228</td>
<td>54</td>
<td>130/70</td>
<td>39.47</td>
<td>9.0</td>
<td>572</td>
</tr>
<tr>
<td>115</td>
<td>38</td>
<td>167</td>
<td>243.6</td>
<td>31</td>
<td>130/80</td>
<td>39.68</td>
<td>6.0</td>
<td>375</td>
</tr>
<tr>
<td>118</td>
<td>48.5</td>
<td>120</td>
<td>359</td>
<td>53.79</td>
<td>100/70</td>
<td>22.28</td>
<td>6.0</td>
<td>287</td>
</tr>
<tr>
<td>202</td>
<td>22.8</td>
<td>176</td>
<td>97</td>
<td>33.3</td>
<td>150/80</td>
<td>106.53</td>
<td>7.0</td>
<td>435</td>
</tr>
<tr>
<td>204</td>
<td>38.3</td>
<td>242</td>
<td>307</td>
<td>40.5</td>
<td>140/90</td>
<td>34.74</td>
<td>7.0</td>
<td>462</td>
</tr>
<tr>
<td>301</td>
<td>45.1</td>
<td>168</td>
<td>251</td>
<td>43</td>
<td>130/80</td>
<td>38.51</td>
<td>7.0</td>
<td>231</td>
</tr>
<tr>
<td>302</td>
<td>72.8</td>
<td>108</td>
<td>287</td>
<td>59.3</td>
<td>130/90</td>
<td>36</td>
<td>8.0</td>
<td>253</td>
</tr>
<tr>
<td>305</td>
<td>30.9</td>
<td>254</td>
<td>214</td>
<td>80.2</td>
<td>180/100</td>
<td>59.19</td>
<td>4.0</td>
<td>263</td>
</tr>
<tr>
<td>306</td>
<td>51.9</td>
<td>111</td>
<td>206.3</td>
<td>59.5</td>
<td>160/100</td>
<td>58.17</td>
<td>4.0</td>
<td>190</td>
</tr>
<tr>
<td>404</td>
<td>29.5</td>
<td>166</td>
<td>189</td>
<td>72.9</td>
<td>115/70</td>
<td>44.97</td>
<td>4.5</td>
<td>239</td>
</tr>
<tr>
<td>406</td>
<td>51.2</td>
<td>138</td>
<td>270</td>
<td>134</td>
<td>120/80</td>
<td>34.57</td>
<td>6.5</td>
<td>113</td>
</tr>
<tr>
<td>407</td>
<td>24.4</td>
<td>133</td>
<td>124.4</td>
<td>53</td>
<td>140/90</td>
<td>85.74</td>
<td>6.5</td>
<td>357</td>
</tr>
<tr>
<td>408</td>
<td>22</td>
<td>264</td>
<td>161.7</td>
<td>29.5</td>
<td>200/90</td>
<td>78.33</td>
<td>6.5</td>
<td>157</td>
</tr>
<tr>
<td>409</td>
<td>45.3</td>
<td>126</td>
<td>247.3</td>
<td></td>
<td>140/90</td>
<td>43.13</td>
<td>4.5</td>
<td>424</td>
</tr>
<tr>
<td>411</td>
<td>38.5</td>
<td>113</td>
<td>144</td>
<td>39</td>
<td>140/80</td>
<td>69.44</td>
<td>4.0</td>
<td>519</td>
</tr>
<tr>
<td>413</td>
<td>69.1</td>
<td>92</td>
<td>359</td>
<td>100</td>
<td>110/80</td>
<td>25.07</td>
<td>5.5</td>
<td>156</td>
</tr>
<tr>
<td>415</td>
<td>36.4</td>
<td>163</td>
<td>201</td>
<td>45.5</td>
<td>130/80</td>
<td>48.09</td>
<td>4.0</td>
<td>108</td>
</tr>
</tbody>
</table>
### APPENDIX V

**Patients who withdrew from trial - all started GR321918**

<table>
<thead>
<tr>
<th>CODE</th>
<th>1st GFR</th>
<th>1st SeCr</th>
<th>1st ERPF</th>
<th>1st ClCr</th>
<th>1st BP</th>
<th>1st RVR</th>
<th>1st CyA dose</th>
<th>1st CyA level</th>
</tr>
</thead>
<tbody>
<tr>
<td>110</td>
<td>45.6</td>
<td>108</td>
<td>226</td>
<td>75.5</td>
<td>140/90</td>
<td>47.19</td>
<td>6.6</td>
<td>257</td>
</tr>
<tr>
<td>116</td>
<td>29.9</td>
<td>151</td>
<td>122</td>
<td>33.3</td>
<td>120/70</td>
<td>71.03</td>
<td>6.0</td>
<td>350</td>
</tr>
<tr>
<td>205</td>
<td>27.9</td>
<td>194</td>
<td>187</td>
<td>33.9</td>
<td>130/70</td>
<td>48.13</td>
<td>4.5</td>
<td>442</td>
</tr>
<tr>
<td>208</td>
<td>47.9</td>
<td>135</td>
<td>127.2</td>
<td>61.9</td>
<td>120/90</td>
<td>78.61</td>
<td>7.0</td>
<td>245</td>
</tr>
</tbody>
</table>
APPENDIX VI

Results from GR32191B trial  (Section 4.3)

1  GR32191B Groups 1+2+3+4

<table>
<thead>
<tr>
<th></th>
<th>PRE-</th>
<th>POST-GR32191B</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP(s)</td>
<td>138 (22.1)</td>
<td>136.9 (22.8)</td>
</tr>
<tr>
<td>BP(d)</td>
<td>85 (15.7)</td>
<td>83.8 (9.2)</td>
</tr>
<tr>
<td>RVR</td>
<td>46.4 (15.3)</td>
<td>51.82 (20.6)</td>
</tr>
</tbody>
</table>

2  GR32191B Groups 1+2+3+4

<table>
<thead>
<tr>
<th></th>
<th>PRE-</th>
<th>POST-GR32191B</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYA dose</td>
<td>6.11 (1.85)</td>
<td>5.98 (1.59)</td>
</tr>
<tr>
<td>CYA level</td>
<td>304.9 (109.71)</td>
<td>301.3 (141.59)</td>
</tr>
</tbody>
</table>

3  GR32191B Group 1

<table>
<thead>
<tr>
<th></th>
<th>PRE-</th>
<th>POST-GR32191B</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP(s)</td>
<td>132.8 (27)</td>
<td>127.1 (16)</td>
</tr>
<tr>
<td>BP(d)</td>
<td>83.6 (18.9)</td>
<td>80 (10)</td>
</tr>
<tr>
<td>RVR</td>
<td>41.2 (18.5)</td>
<td>46.5 (14)</td>
</tr>
</tbody>
</table>

4  GR32191B Groups 2+3

<table>
<thead>
<tr>
<th></th>
<th>PRE-</th>
<th>POST-GR32191B</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYA dose</td>
<td>5.75 (1.41)</td>
<td>6.17 (1.72)</td>
</tr>
<tr>
<td>CYA trough</td>
<td>311.8 (78.04)</td>
<td>305.7 (113.77)</td>
</tr>
</tbody>
</table>

5  GR32191B Group 4

<table>
<thead>
<tr>
<th></th>
<th>PRE-</th>
<th>POST-GR32191B</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP(s)</td>
<td>141 (20.6)</td>
<td>136.9 (28.2)</td>
</tr>
<tr>
<td>BP(d)</td>
<td>87.5 (12.8)</td>
<td>82.5 (7.1)</td>
</tr>
<tr>
<td>RVR</td>
<td>50.1 (14)</td>
<td>53.7 (21.7)</td>
</tr>
</tbody>
</table>

6  GR32191B Groups 2+3+4

<table>
<thead>
<tr>
<th></th>
<th>PRE-</th>
<th>POST-GR32191B</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYA dose</td>
<td>5.46 (1.24)</td>
<td>5.64 (1.43)</td>
</tr>
<tr>
<td>CYA trough</td>
<td>300.6 (89.07)</td>
<td>279.1 (136.5)</td>
</tr>
</tbody>
</table>
**APPENDIX VII**

**PROSTAGLANDINS**

Urinary prostanoid concentrations in patients receiving PLACEBO (ng/24 hour)

<table>
<thead>
<tr>
<th>CODE</th>
<th>1st TXB₂</th>
<th>TXB₂ after 3/12 Rx</th>
<th>TXB₂ 6/12 later</th>
<th>1st 6kPGF₁α</th>
<th>6kPGF₁α after 3/12 Rx</th>
<th>6kPGF₁α 6/12 later</th>
</tr>
</thead>
<tbody>
<tr>
<td>102</td>
<td>870</td>
<td>3480</td>
<td>2165</td>
<td>1097</td>
<td>988</td>
<td>1410</td>
</tr>
<tr>
<td>104</td>
<td>1670</td>
<td>7608</td>
<td>2167</td>
<td>1481</td>
<td>1591</td>
<td>839</td>
</tr>
<tr>
<td>105</td>
<td>2487</td>
<td>4950</td>
<td>1114</td>
<td>1987</td>
<td>1448</td>
<td>590</td>
</tr>
<tr>
<td>108</td>
<td>876</td>
<td>1620</td>
<td>739</td>
<td>298</td>
<td>524</td>
<td>971</td>
</tr>
<tr>
<td>109</td>
<td>820</td>
<td>864</td>
<td>1156</td>
<td>596</td>
<td>733</td>
<td>571</td>
</tr>
<tr>
<td>111</td>
<td>2754</td>
<td>2604</td>
<td></td>
<td>1260</td>
<td>791</td>
<td></td>
</tr>
<tr>
<td>113</td>
<td>1707</td>
<td>1968</td>
<td></td>
<td>1707</td>
<td>756</td>
<td></td>
</tr>
<tr>
<td>114</td>
<td>2450</td>
<td>2568</td>
<td></td>
<td>2450</td>
<td>683</td>
<td></td>
</tr>
<tr>
<td>201</td>
<td>2178</td>
<td>2066</td>
<td>762</td>
<td>2096</td>
<td>698</td>
<td>552</td>
</tr>
<tr>
<td>203</td>
<td>1666</td>
<td>3300</td>
<td>2539</td>
<td>1700</td>
<td>1948</td>
<td>1145</td>
</tr>
<tr>
<td>206</td>
<td>2100</td>
<td>1331</td>
<td>1185</td>
<td>698</td>
<td>842</td>
<td>1062</td>
</tr>
<tr>
<td>207</td>
<td>1509</td>
<td>5775</td>
<td>3359</td>
<td>896</td>
<td>1408</td>
<td>1068</td>
</tr>
<tr>
<td>303</td>
<td>1270</td>
<td>4000</td>
<td>2255</td>
<td>909</td>
<td>1468</td>
<td>625</td>
</tr>
<tr>
<td>304</td>
<td>3574</td>
<td>4043</td>
<td>840</td>
<td>2007</td>
<td>1490</td>
<td>1430</td>
</tr>
<tr>
<td>401</td>
<td>3308</td>
<td>5250</td>
<td>2674</td>
<td>1906</td>
<td>709</td>
<td>1285</td>
</tr>
<tr>
<td>402</td>
<td>2835</td>
<td>2695</td>
<td>3839</td>
<td>1609</td>
<td>1503</td>
<td>878</td>
</tr>
<tr>
<td>403</td>
<td>2106</td>
<td>2250</td>
<td></td>
<td>1107</td>
<td>1309</td>
<td></td>
</tr>
<tr>
<td>405</td>
<td>876</td>
<td>1348</td>
<td>1088</td>
<td>916</td>
<td>640</td>
<td>988</td>
</tr>
<tr>
<td>410</td>
<td>1939</td>
<td>5000</td>
<td>2232</td>
<td>914</td>
<td>1790</td>
<td>565</td>
</tr>
<tr>
<td>412</td>
<td>1249</td>
<td>1610</td>
<td>660</td>
<td>816</td>
<td>1090</td>
<td>554</td>
</tr>
<tr>
<td>414</td>
<td>1656</td>
<td>2325</td>
<td>1651</td>
<td>798</td>
<td>1290</td>
<td>753</td>
</tr>
<tr>
<td>CODE</td>
<td>1st TXB$_2$</td>
<td>TXB$_2$ after 3/12 Rx</td>
<td>TXB$_2$ 6/12 later</td>
<td>1st 6kPGF$_{1a}$</td>
<td>6kPGF$_{1a}$ after 3/12 Rx</td>
<td>6kPGF$_{1a}$ 6/12 later</td>
</tr>
<tr>
<td>------</td>
<td>-------------</td>
<td>------------------------</td>
<td>---------------------</td>
<td>----------------</td>
<td>-----------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>101</td>
<td>786</td>
<td>3580</td>
<td>989</td>
<td>1386</td>
<td>1107</td>
<td>874</td>
</tr>
<tr>
<td>103</td>
<td>1501</td>
<td>3780</td>
<td>1831</td>
<td>1510</td>
<td>1289</td>
<td>1007</td>
</tr>
<tr>
<td>106</td>
<td>2341</td>
<td>2591</td>
<td>1360</td>
<td>1691</td>
<td>1498</td>
<td>717</td>
</tr>
<tr>
<td>107</td>
<td>4101</td>
<td>6574</td>
<td>1396</td>
<td>2408</td>
<td>1390</td>
<td>915</td>
</tr>
<tr>
<td>110</td>
<td>4188</td>
<td>1712</td>
<td></td>
<td>989</td>
<td></td>
<td></td>
</tr>
<tr>
<td>112</td>
<td>2532</td>
<td>1284</td>
<td></td>
<td>1108</td>
<td>526</td>
<td></td>
</tr>
<tr>
<td>115</td>
<td>2333</td>
<td>1236</td>
<td></td>
<td>2333</td>
<td>501</td>
<td></td>
</tr>
<tr>
<td>202</td>
<td>934</td>
<td>1760</td>
<td>2090</td>
<td>1288</td>
<td>1198</td>
<td>1274</td>
</tr>
<tr>
<td>204</td>
<td>1071</td>
<td>2350</td>
<td>1907</td>
<td>907</td>
<td>1006</td>
<td>2924</td>
</tr>
<tr>
<td>205</td>
<td>1236</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>301</td>
<td>821</td>
<td>2460</td>
<td>919</td>
<td>1490</td>
<td>989</td>
<td>1070</td>
</tr>
<tr>
<td>302</td>
<td>2116</td>
<td>1400</td>
<td></td>
<td>1099</td>
<td>1360</td>
<td></td>
</tr>
<tr>
<td>305</td>
<td>1242</td>
<td>5016</td>
<td>2086</td>
<td>896</td>
<td>1896</td>
<td>572</td>
</tr>
<tr>
<td>404</td>
<td>1356</td>
<td>2580</td>
<td>1428</td>
<td>942</td>
<td>1861</td>
<td>705</td>
</tr>
<tr>
<td>406</td>
<td>2025</td>
<td>4756</td>
<td>2697</td>
<td>1104</td>
<td>990</td>
<td>656</td>
</tr>
<tr>
<td>407</td>
<td>1222</td>
<td>2652</td>
<td>1058</td>
<td>1006</td>
<td>1200</td>
<td>440</td>
</tr>
<tr>
<td>408</td>
<td>1344</td>
<td>1596</td>
<td></td>
<td>846</td>
<td>490</td>
<td></td>
</tr>
<tr>
<td>409</td>
<td>3162</td>
<td>1534</td>
<td>1304</td>
<td>1900</td>
<td>698</td>
<td>641</td>
</tr>
<tr>
<td>411</td>
<td>1728</td>
<td>1875</td>
<td>1909</td>
<td>1261</td>
<td>1106</td>
<td>735</td>
</tr>
<tr>
<td>413</td>
<td>3480</td>
<td>2168</td>
<td></td>
<td>1206</td>
<td>960</td>
<td></td>
</tr>
<tr>
<td>415</td>
<td>2438</td>
<td>2372</td>
<td>1708</td>
<td>1696</td>
<td>838</td>
<td>701</td>
</tr>
</tbody>
</table>
Normal ranges for urinary prostanoids

measured from healthy volunteers (Whiting, P. H. -personal communication)

\[ \text{TXB}_2 \text{ ng/24hr} \quad n = 25 \quad 22-56 \text{ yr} \quad 14F:11M \]

\[ 380-940 \quad \text{mean} \; 610 \; (+/-214) \text{ ng/24hr} \]

\[ 6k\text{PGF}_{1\alpha} \text{ ng/24hr} \] same group as above

\[ 650-1550 \quad \text{mean} \; 1194 \; (+/0316) \text{ ng/24hr} \]

[Neither value showed any relationship to age or sex]
### APPENDIX VIII

**Plasma prostanoids in patients receiving PLACEBO (pg/ml)**

<table>
<thead>
<tr>
<th>CODE</th>
<th>1st TXB₂</th>
<th>TXB₂ after Rₓ</th>
<th>1st 6kPGF₁a</th>
<th>6kPGF₁a after Rₓ</th>
</tr>
</thead>
<tbody>
<tr>
<td>102</td>
<td>106</td>
<td>125</td>
<td>2.43</td>
<td>2.46</td>
</tr>
<tr>
<td>104</td>
<td>169</td>
<td>129</td>
<td>2.18</td>
<td>3.59</td>
</tr>
<tr>
<td>105</td>
<td>396</td>
<td>243</td>
<td>2.36</td>
<td>1.61</td>
</tr>
<tr>
<td>201</td>
<td>74</td>
<td>93</td>
<td>2.86</td>
<td>1.9</td>
</tr>
<tr>
<td>203</td>
<td>102</td>
<td>52</td>
<td>2.24</td>
<td>1.5</td>
</tr>
<tr>
<td>206</td>
<td>103</td>
<td>277</td>
<td>2.96</td>
<td>2.33</td>
</tr>
<tr>
<td>303</td>
<td>59</td>
<td>89</td>
<td>1.82</td>
<td>2.84</td>
</tr>
<tr>
<td>304</td>
<td>117</td>
<td>204</td>
<td>3.04</td>
<td>1.81</td>
</tr>
<tr>
<td>401</td>
<td>22</td>
<td>41</td>
<td>1.3</td>
<td>3.71</td>
</tr>
<tr>
<td>402</td>
<td>150</td>
<td>135</td>
<td>2.43</td>
<td>3.17</td>
</tr>
<tr>
<td>403</td>
<td>74</td>
<td>71</td>
<td>2.53</td>
<td>2.1</td>
</tr>
<tr>
<td>405</td>
<td>281</td>
<td>141</td>
<td>4.02</td>
<td>3.91</td>
</tr>
</tbody>
</table>

**Plasma prostanoids in patients receiving GR32191B (pg/ml)**

<table>
<thead>
<tr>
<th>CODE</th>
<th>1st TXB₂</th>
<th>TXB₂ after Rₓ</th>
<th>1st 6kPGF₁a</th>
<th>6kPGF₁a after Rₓ</th>
</tr>
</thead>
<tbody>
<tr>
<td>101</td>
<td>157</td>
<td>94</td>
<td>4.69</td>
<td>1.87</td>
</tr>
<tr>
<td>103</td>
<td>122</td>
<td>110</td>
<td>2.8</td>
<td>2.2</td>
</tr>
<tr>
<td>202</td>
<td>69</td>
<td>81</td>
<td>2.37</td>
<td>1.8</td>
</tr>
<tr>
<td>204</td>
<td>55</td>
<td>103</td>
<td>1.76</td>
<td>1.62</td>
</tr>
<tr>
<td>301</td>
<td>77</td>
<td>373</td>
<td>2.08</td>
<td>1.32</td>
</tr>
<tr>
<td>302</td>
<td>107</td>
<td>153</td>
<td>2.47</td>
<td>1.7</td>
</tr>
<tr>
<td>404</td>
<td>117</td>
<td>94</td>
<td>1.96</td>
<td>2.24</td>
</tr>
</tbody>
</table>
APPENDIX IX

Urinary biochemistry and NAG

ALL PATIENTS - PLACEBO GROUP

<table>
<thead>
<tr>
<th></th>
<th>PRE-</th>
<th>POST-PLACEBO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary Glucose</td>
<td>1.25 (2.06)</td>
<td>0.93 (1.7)</td>
</tr>
<tr>
<td>Fe Na %</td>
<td>1.73 (1.09)</td>
<td>1.27 (0.86)</td>
</tr>
<tr>
<td>Urinary Na</td>
<td>78.57 (37.45)</td>
<td>71.9 (34.27)</td>
</tr>
<tr>
<td>Urinary K</td>
<td>30.95 (11.37)</td>
<td>29.19 (11.91)</td>
</tr>
<tr>
<td>NAG</td>
<td>59.18 (51.82)</td>
<td>68.71 (116.73)</td>
</tr>
</tbody>
</table>

* p = 0.025

ALL PATIENTS - GR32191B GROUP

<table>
<thead>
<tr>
<th></th>
<th>PRE-</th>
<th>POST-GR32191B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary Glucose</td>
<td>0.62 (1.04)</td>
<td>0.55 (0.96)</td>
</tr>
<tr>
<td>Fe Na %</td>
<td>1.79 (0.87)</td>
<td>1.6 (0.75)</td>
</tr>
<tr>
<td>Urinary Na</td>
<td>84.84 (26.88)</td>
<td>76.21 (30.34)</td>
</tr>
<tr>
<td>Urinary K</td>
<td>33.32 (16.08)</td>
<td>33.42 (21.41)</td>
</tr>
<tr>
<td>NAG</td>
<td>61.77 (54.94)</td>
<td>46.95 (35.13)</td>
</tr>
</tbody>
</table>

Normal ranges

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary Glucose</td>
<td>&lt; 0.5mmol/mmol Cr</td>
</tr>
<tr>
<td>FeNa %</td>
<td>0.6 to 1.6%</td>
</tr>
<tr>
<td>Urinary Na</td>
<td>80-200mmol/d</td>
</tr>
<tr>
<td>Urinary K</td>
<td>30-100mmol/d</td>
</tr>
<tr>
<td>NAG</td>
<td>&lt; 35 IU/mmol Cr</td>
</tr>
</tbody>
</table>
APPENDIX X

ACUTE STUDY

Renal allograft recipients after a therapeutic dose of CyA

<table>
<thead>
<tr>
<th>TIME</th>
<th>GFR</th>
<th>ERPF</th>
<th>BP</th>
<th>FF</th>
<th>RVR</th>
<th>FR</th>
<th>CyA</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>159</td>
</tr>
<tr>
<td>140</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CyA-</td>
<td>210</td>
<td>50.7</td>
<td>254</td>
<td>110/80</td>
<td>19.8</td>
<td>38.3</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>270</td>
<td>46.5</td>
<td>235</td>
<td>110/90</td>
<td>19.7</td>
<td>41.1</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>55.3</td>
<td>268</td>
<td>120/90</td>
<td>20.7</td>
<td>37.3</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>390</td>
<td>55.6</td>
<td>272</td>
<td>110/90</td>
<td>20.5</td>
<td>35.6</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>450</td>
<td>55.5</td>
<td>285</td>
<td>110/90</td>
<td>19.5</td>
<td>33.9</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>510</td>
<td>56.0</td>
<td>278</td>
<td>110/90</td>
<td>20.2</td>
<td>34.8</td>
<td>6.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TIME</th>
<th>GFR</th>
<th>ERPF</th>
<th>BP</th>
<th>FF</th>
<th>RVR</th>
<th>FR</th>
<th>CyA</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>130/100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>45.0</td>
<td>222</td>
<td>130/100</td>
<td>20.3</td>
<td>49.6</td>
<td>6.4</td>
<td>166</td>
</tr>
<tr>
<td>CyA-</td>
<td>180</td>
<td>34.7</td>
<td>149</td>
<td>130/100</td>
<td>23.2</td>
<td>73.9</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>37.6</td>
<td>156</td>
<td>140/100</td>
<td>24.1</td>
<td>72.7</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>41.0</td>
<td>173</td>
<td>140/90</td>
<td>23.6</td>
<td>61.5</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>360</td>
<td>39.8</td>
<td>166</td>
<td>130/90</td>
<td>24.0</td>
<td>62.3</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>420</td>
<td>35.6</td>
<td>147</td>
<td>140/90</td>
<td>24.2</td>
<td>77.1</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>480</td>
<td>40.5</td>
<td>167</td>
<td>140/100</td>
<td>24.2</td>
<td>67.9</td>
<td>6.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TIME</th>
<th>GFR</th>
<th>ERPF</th>
<th>BP</th>
<th>FF</th>
<th>RVR</th>
<th>FR</th>
<th>CyA</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>140/90</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>149</td>
</tr>
<tr>
<td>120</td>
<td>40.0</td>
<td>143</td>
<td>180/90</td>
<td>28.1</td>
<td>84.2</td>
<td>11.0</td>
<td>153</td>
</tr>
<tr>
<td>CyA-</td>
<td>180</td>
<td>36.5</td>
<td>140</td>
<td>150/90</td>
<td>26.1</td>
<td>78.9</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>37.6</td>
<td>145</td>
<td>140/70</td>
<td>26.0</td>
<td>64.5</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>35.0</td>
<td>137</td>
<td>160/90</td>
<td>25.5</td>
<td>82.6</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>360</td>
<td>39.4</td>
<td>143</td>
<td>140/80</td>
<td>27.6</td>
<td>7.0</td>
<td>8.2</td>
</tr>
<tr>
<td></td>
<td>420</td>
<td>37.2</td>
<td>142</td>
<td>160/90</td>
<td>26.1</td>
<td>79.6</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>480</td>
<td>38.9</td>
<td>145</td>
<td>170/90</td>
<td>26.8</td>
<td>80.4</td>
<td>6.7</td>
</tr>
<tr>
<td>TIME</td>
<td>GFR</td>
<td>ERPF</td>
<td>BP</td>
<td>FF</td>
<td>RVR</td>
<td>FR</td>
<td>CyA</td>
</tr>
<tr>
<td>------</td>
<td>-----</td>
<td>------</td>
<td>-------</td>
<td>----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>60</td>
<td>105/80</td>
<td>204</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>110</td>
<td>63.8</td>
<td>317</td>
<td>100/80</td>
<td>20.1</td>
<td>27.3</td>
<td>8.6</td>
<td>197</td>
</tr>
<tr>
<td>CyA-→ 165</td>
<td>54.1</td>
<td>259</td>
<td>110/60</td>
<td>20.9</td>
<td>29.7</td>
<td>8.2</td>
<td>177</td>
</tr>
<tr>
<td>225</td>
<td>73.1</td>
<td>354</td>
<td>110/60</td>
<td>20.6</td>
<td>21.6</td>
<td>5.4</td>
<td>226</td>
</tr>
<tr>
<td>285</td>
<td>78.1</td>
<td>359</td>
<td>100/70</td>
<td>21.8</td>
<td>22.3</td>
<td>3.8</td>
<td>200</td>
</tr>
<tr>
<td>345</td>
<td>58.9</td>
<td>279</td>
<td>110/80</td>
<td>21.1</td>
<td>32.3</td>
<td>6.4</td>
<td>307</td>
</tr>
<tr>
<td>405</td>
<td>58.4</td>
<td>266</td>
<td>90/55</td>
<td>21.9</td>
<td>25.0</td>
<td>3.4</td>
<td>334</td>
</tr>
<tr>
<td>465</td>
<td>58.9</td>
<td>258</td>
<td>90/55</td>
<td>22.8</td>
<td>25.8</td>
<td>3.9</td>
<td>275</td>
</tr>
</tbody>
</table>

**Dermatology patients following acute dosing with 12mg/kg of CyA**

<table>
<thead>
<tr>
<th>TIME</th>
<th>GFR</th>
<th>ERPF</th>
<th>BP</th>
<th>FF</th>
<th>RVR</th>
<th>FR</th>
<th>CyA</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>120/60</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>135</td>
<td>141</td>
<td>598</td>
<td>130/80</td>
<td>23.5</td>
<td>16.2</td>
<td>12.4</td>
<td>0</td>
</tr>
<tr>
<td>CyA-→ 180</td>
<td>97</td>
<td>401</td>
<td>120/90</td>
<td>24.0</td>
<td>24.9</td>
<td>11.2</td>
<td>0</td>
</tr>
<tr>
<td>225</td>
<td>98</td>
<td>407</td>
<td>125/80</td>
<td>24.1</td>
<td>23.3</td>
<td>11.7</td>
<td>394</td>
</tr>
<tr>
<td>270</td>
<td>99</td>
<td>410</td>
<td>130/70</td>
<td>24.2</td>
<td>22.7</td>
<td>11.9</td>
<td>671</td>
</tr>
<tr>
<td>315</td>
<td>99</td>
<td>401</td>
<td>120/80</td>
<td>24.8</td>
<td>23.3</td>
<td>11.1</td>
<td>909</td>
</tr>
<tr>
<td>375</td>
<td>-</td>
<td>-</td>
<td>130/80</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>690</td>
</tr>
<tr>
<td>425</td>
<td>107</td>
<td>418</td>
<td>125/85</td>
<td>25.6</td>
<td>23.5</td>
<td>7.1</td>
<td>544</td>
</tr>
<tr>
<td>495</td>
<td>106</td>
<td>413</td>
<td>130/95</td>
<td>25.6</td>
<td>25.8</td>
<td>6.5</td>
<td>423</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TIME</th>
<th>GFR</th>
<th>ERPF</th>
<th>BP</th>
<th>FF</th>
<th>RVR</th>
<th>FR</th>
<th>CyA</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>103</td>
<td>106</td>
<td>496</td>
<td>140/80</td>
<td>21.4</td>
<td>20.9</td>
<td>14.7</td>
<td>0</td>
</tr>
<tr>
<td>CyA-→ 148</td>
<td>99</td>
<td>449</td>
<td>140/80</td>
<td>22.0</td>
<td>23.0</td>
<td>15.2</td>
<td>0</td>
</tr>
<tr>
<td>193</td>
<td>114</td>
<td>509</td>
<td>160/90</td>
<td>22.4</td>
<td>22.3</td>
<td>14.7</td>
<td>584</td>
</tr>
<tr>
<td>238</td>
<td>115</td>
<td>501</td>
<td>155/85</td>
<td>22.9</td>
<td>21.6</td>
<td>10.4</td>
<td>726</td>
</tr>
<tr>
<td>283</td>
<td>123</td>
<td>513</td>
<td>150/80</td>
<td>24.0</td>
<td>20.8</td>
<td>5.6</td>
<td>680</td>
</tr>
<tr>
<td>328</td>
<td>119</td>
<td>483</td>
<td>150/85</td>
<td>24.5</td>
<td>22.1</td>
<td>14.8</td>
<td>563</td>
</tr>
<tr>
<td>373</td>
<td>116</td>
<td>475</td>
<td>150/90</td>
<td>24.5</td>
<td>23.2</td>
<td>12.3</td>
<td>395</td>
</tr>
<tr>
<td>418</td>
<td>118</td>
<td>477</td>
<td>145/90</td>
<td>24.7</td>
<td>22.7</td>
<td>12.5</td>
<td>403</td>
</tr>
<tr>
<td>463</td>
<td>119</td>
<td>496</td>
<td>140/100</td>
<td>24.0</td>
<td>22.8</td>
<td>10.9</td>
<td>381</td>
</tr>
</tbody>
</table>
### GP

<table>
<thead>
<tr>
<th>TIME</th>
<th>GFR</th>
<th>ERPF</th>
<th>BP</th>
<th>FF</th>
<th>RVR</th>
<th>FR</th>
<th>CyA</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td></td>
<td></td>
<td>110/80</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>134</td>
<td>531</td>
<td>110/70</td>
<td>25.2</td>
<td>15.7</td>
<td>8.5</td>
<td>0</td>
</tr>
<tr>
<td>CyA-→</td>
<td>195</td>
<td>131</td>
<td>535</td>
<td>115/70</td>
<td>24.4</td>
<td>15.9</td>
<td>20.4</td>
</tr>
<tr>
<td>255</td>
<td>118</td>
<td>453</td>
<td>120/70</td>
<td>26.1</td>
<td>19.1</td>
<td>17.0</td>
<td>642</td>
</tr>
<tr>
<td>315</td>
<td>131</td>
<td>499</td>
<td>120/80</td>
<td>26.2</td>
<td>18.7</td>
<td>11.5</td>
<td>1134</td>
</tr>
<tr>
<td>375</td>
<td>128</td>
<td>482</td>
<td>120/80</td>
<td>26.4</td>
<td>20.0</td>
<td>10.6</td>
<td>1067</td>
</tr>
<tr>
<td>435</td>
<td>131</td>
<td>461</td>
<td>130/80</td>
<td>28.4</td>
<td>21.0</td>
<td>7.3</td>
<td>804</td>
</tr>
<tr>
<td>495</td>
<td>138</td>
<td>465</td>
<td>130/80</td>
<td>30.0</td>
<td>20.8</td>
<td>10.0</td>
<td>742</td>
</tr>
</tbody>
</table>

### IA

<table>
<thead>
<tr>
<th>TIME</th>
<th>GFR</th>
<th>ERPF</th>
<th>BP</th>
<th>FF</th>
<th>RVR</th>
<th>FR</th>
<th>CyA</th>
</tr>
</thead>
<tbody>
<tr>
<td>75</td>
<td></td>
<td></td>
<td>120/80</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>135</td>
<td>116</td>
<td>514</td>
<td>110/80</td>
<td>22.5</td>
<td>17.5</td>
<td>14.0</td>
<td>0</td>
</tr>
<tr>
<td>CyA-→</td>
<td>195</td>
<td>118</td>
<td>501</td>
<td>110/70</td>
<td>23.4</td>
<td>16.6</td>
<td>12.5</td>
</tr>
<tr>
<td>255</td>
<td>116</td>
<td>484</td>
<td>110/80</td>
<td>23.9</td>
<td>18.6</td>
<td>10.9</td>
<td>783</td>
</tr>
<tr>
<td>315</td>
<td>122</td>
<td>473</td>
<td>110/80</td>
<td>25.8</td>
<td>18.3</td>
<td>9.8</td>
<td>1044</td>
</tr>
<tr>
<td>375</td>
<td>130</td>
<td>500</td>
<td>110/80</td>
<td>26.0</td>
<td>18.0</td>
<td>10.3</td>
<td>540</td>
</tr>
<tr>
<td>435</td>
<td>112</td>
<td>437</td>
<td>110/85</td>
<td>25.7</td>
<td>21.3</td>
<td>10.3</td>
<td>403</td>
</tr>
<tr>
<td>495</td>
<td>118</td>
<td>454</td>
<td>110/80</td>
<td>25.9</td>
<td>19.9</td>
<td>10.3</td>
<td>463</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TIME</th>
<th>min</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFR</td>
<td>ml/min</td>
</tr>
<tr>
<td>ERPF</td>
<td>ml/min</td>
</tr>
<tr>
<td>BP</td>
<td>mm Hg</td>
</tr>
<tr>
<td>FF</td>
<td>%</td>
</tr>
<tr>
<td>RVR</td>
<td>dyne sec/cm²</td>
</tr>
<tr>
<td>FR</td>
<td>ml/min</td>
</tr>
<tr>
<td>CyA</td>
<td>ng/ml</td>
</tr>
</tbody>
</table>
# APPENDIX XI

## RENAL FUNCTIONAL RESERVE

Before and after a 93g oral protein meal

### Glomerular Filtration Rate (ml/min)

<table>
<thead>
<tr>
<th>PT No</th>
<th>BASELINE</th>
<th>POST-PRANDIAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hour</td>
<td>2 hours</td>
</tr>
<tr>
<td>18</td>
<td>22.6</td>
<td>19.2</td>
</tr>
<tr>
<td>17</td>
<td>37.3</td>
<td>38.0</td>
</tr>
<tr>
<td>8</td>
<td>40.1</td>
<td>41.4</td>
</tr>
<tr>
<td>33</td>
<td>41.7</td>
<td>39.6</td>
</tr>
<tr>
<td>6</td>
<td>43</td>
<td>37.9</td>
</tr>
<tr>
<td>7</td>
<td>50.0</td>
<td>50.6</td>
</tr>
<tr>
<td>32</td>
<td>65.5</td>
<td>58.8</td>
</tr>
<tr>
<td>11</td>
<td>70.3</td>
<td>66.6</td>
</tr>
<tr>
<td>12</td>
<td>92.8</td>
<td>86.1</td>
</tr>
</tbody>
</table>

### Effective Renal Plasma Flow (ml/min)

<table>
<thead>
<tr>
<th>PT No</th>
<th>BASELINE</th>
<th>POST-PRANDIAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hour</td>
<td>2 hours</td>
</tr>
<tr>
<td>18</td>
<td>61.9</td>
<td>54.4</td>
</tr>
<tr>
<td>17</td>
<td>126.7</td>
<td>121.8</td>
</tr>
<tr>
<td>8</td>
<td>153.2</td>
<td>165.8</td>
</tr>
<tr>
<td>33</td>
<td>260.0</td>
<td>236.0</td>
</tr>
<tr>
<td>6</td>
<td>203.6</td>
<td>182.1</td>
</tr>
<tr>
<td>7</td>
<td>210.5</td>
<td>203.6</td>
</tr>
<tr>
<td>32</td>
<td>235.4</td>
<td>217.1</td>
</tr>
<tr>
<td>11</td>
<td>294.4</td>
<td>263.8</td>
</tr>
<tr>
<td>12</td>
<td>404.5</td>
<td>367.7</td>
</tr>
</tbody>
</table>

### Urinary Flow Rates (ml/min)

<table>
<thead>
<tr>
<th>PT No</th>
<th>BASELINE</th>
<th>POST-PRANDIAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hour</td>
<td>2 hours</td>
</tr>
<tr>
<td>18</td>
<td>3.7</td>
<td>2.5</td>
</tr>
<tr>
<td>17</td>
<td>7.5</td>
<td>8.0</td>
</tr>
<tr>
<td>8</td>
<td>14.2</td>
<td>15.0</td>
</tr>
<tr>
<td>33</td>
<td>8.9</td>
<td>9.1</td>
</tr>
<tr>
<td>6</td>
<td>10.0</td>
<td>7.0</td>
</tr>
<tr>
<td>7</td>
<td>6.2</td>
<td>6.8</td>
</tr>
<tr>
<td>32</td>
<td>7.2</td>
<td>6.8</td>
</tr>
<tr>
<td>11</td>
<td>6.8</td>
<td>8.7</td>
</tr>
<tr>
<td>12</td>
<td>12.3</td>
<td>10.4</td>
</tr>
</tbody>
</table>
Tromboxane receptor antagonist in renal transplantation.
Transplantation Proceedings (in press)

Glomerular hyperfiltration after protein ingestion, during glucagon infusion and in insulin-dependent diabetics is induced by a liver hormone: Deficient production of this hormone in hepatic failure caused hepatorenal syndrome.
The Lancet, i, 195-197.

Single Kidney Function: Effect of Acute Protein and water loading on microalbuminuria.
The American Journal of Medicine, 84, 711-717.

Prostaglandins: Effects on blood pressure, renal blood flow, sodium and water excretion.

Austin, J.H., Stillman, E. & Van Slyke, D.D. (1921)
Factors Governing the excretion rate of urea.
Journal of Biological Chemistry, 46, 91-112.


Effects of Cyclosporine on the Renin-Angiotensin Aldosterone System and Potassium Excretion in Renal Transplant recipients.
Archives of Internal Medicine, 145, 505-508.

Clinical appraisal of creatinine clearance as a measurement of glomerular filtration rate.

Interaction of the prostaglandin and renin-angiotensin systems in isolated rat glomeruli.
Urinary excretion of both thromboxane B_2 and its metabolite 2,3-dinor TXB_2 are increased in rats treated with cyclosporin A.

Increased urinary excretion of TXB_2 and 2,3-dinor TXB_2 in cyclosporine A nephrotoxicity.
*Kidney International, 34, 164-174.*

Renal Injury Induced by Long Term Administration of Cyclosporin A to rats.
*American Journal of Pathology, 127, 569-579.*

Bjoro, K. (1986)
Prostacyclin and thromboxane formation in human umbilical arteries following stimulation with vaso-active autacoids.
*Prostaglandins, 31, 699-714.*

Formation of prostanoids in human umbilical vessels perfused in vitro.
*Prostaglandins, 31, 4, 683-698.*

Interstitial fibrosis in human kidney grafts after 12 to 46 months of Cyclosporine therapy.
*Transplantation Proceedings, XVII, No 1, 1168-1171.*

Bonsnes, R.W. & Taussky, H.H. (1945)
On the colorimetric determination of creatinine by the Jaffe reaction.
*Journal of Biological Chemistry, 158, 581-591.*

Short term protein loading in assessment of patients with renal disease.
*The American Journal of Medicine, 77, 873-879.*


Briggs, J.D. (1989)
Cyclosporin nephrotoxicity – what is the problem today?

Cyclosporine inhibits Prostacyclin Production by Cultured Human Endothelial cells.
*Transplantation Proceedings, XIX, No 1, 1178-1180.*
Increased factor VIII as an index of vascular injury in
cyclosporine nephrotoxicity.
*Transplantation, 42*, 150-153.

Post-operative deep venous thrombosis after renal transplantation.
Effects of Cyclosporine.
*Transplantation, 43*, 647-649.

Cyclosporin A mediates immunosuppression of primary cytotoxic T-
cell responses by impairing the release of interleukin-1 and
interleukin-2.
*European Journal of Immunology, 11*, 657-661.

Effect of Verapamil on Glomerular Prostaglandin Production and
glomerular filtration rate during Cyclosporine adminstration.

Glomerular Prostacyclin production.

The Prostacyclin-Thromboxane A_2 balance: Pathophysiological and
Therapeutic Implications.

Failure of cyclosporine-treated renal allograft recipients to
increase glomerular filtration rate following an amino acid
infusion.
*Transplantation, 46*, 79-82.

Calne, R.Y., Thiru, S., McMaster, P., Craddock, G.N., White, D.J.G.,
Cyclosporin A in patients receiving renal allografts from
cadaveric donors.

Cyclosporin in Cadaveric Renal Transplantation: 3 year follow up
of a European Multicentre Trial.
*Lancet, ii*, 549.

Canadian Multicentre Transplant Study Group. (1983)
A Randomised Clinical Trial of Cyclosporine in cadaveric renal
transplantation.
Measurement of Renal and non-renal Eicosanoid synthesis.
The American Journal of Medicine, 81, suppl 2B, 23-29.

Chantler, C. & Barratt, T.M. (1972)
Estimation of Glomerular Filtration Rate from plasma clearance of
51-chromium Edetic Acid.
Archives of Disease in Childhood, 47, 613-617.

Chapman, J.R., Harding, N.G.L., Griffiths, D. & Morris, P.J. (1985)
Reversibility of Cyclosporin nephrotoxicity after three months' treatment.
Lancet, i, 128-129.

Chapman, J.R., Marcen, R., Arias, M., Raine, A.E.G., Dunnill, M.S. &
Morris, P.J. (1987)
Hypertension after renal transplantation; a comparison of
cyclosporin and conventional immunosuppression.
Transplantation, 46, 6, 860-864.

Determination of creatinine by means of automatic chemical analysis.
American Journal of Clinical Pathology, 35, 83-88.

Cibattoni, G., Cinotti, G., Pierucci, A., Simonetti, B.M., Manzi, M.,
Pugliese, F., Barsotti, P., Pecci, G., Taggi, F. & Patrono, C.
(1984)
Effects of Sulindac and Ibuprofen in patients with chronic
glomerular disease.
The New England Journal of Medicine, 310, 5, 279-283.

Claris-Appiani, A., Assael, B.M., Tirelli, A.S., Marra, G. & Cavanna, G.
(1988)
Lack of glomerular hemodynamic stimulation after infusion of branched-chain amino acids.

Prediction of Creatinine Clearance from Serum Creatinine.
Nephron, 16, 31-41.

Evidence that Renal Prostaglandin and Thromboxane Production is stimulated in chronic Cyclosporine Nephrotoxicity.
Transplantation, 43, 2, 282-285.

Constable, A.R., Hussein, M.M., Albrecht, M.P., Thompson, F.D.,
Philalithis, P.E. & Joekes, A.M. (1979)
Single sample estimates of renal clearances.
British Journal of Urology, 51, 84-87.

Constable, A.R., Hussein, M.M., Albrecht, M.P., Thompson, F.D.,
Single sample estimates of renal clearances.
British Journal of Urology, 57, 483.
Cyclosporin in therapeutic doses increases renal allograft vascular resistance.  
Lancet, ii, 477-479.

Renal vasoconstriction in cyclosporin-treated transplant recipients without other evidence of nephrotoxicity.  

The Glomerular Filtration Rate of Stable Renal Transplant Patients receiving Cyclosporine is markedly impaired.  

Renal function studies in cyclosporine-treated non-kidney transplant recipients of pancreas grafts.  
Transplantation Proceedings, XIX, 3995-3997.

Effects of Ciclosporin on plasma renin activity, catecholamines and prostaglandins in patients with idiopathic uveitis.  
American Journal of Nephrology, 8, 298-304.

de Wardener, H.E. (1985)  
The Kidney. An outline of normal and abnormal function.  

Effects of acute protein loads of different sources on glomerular filtration rate.  

Antagonist capacities of nifedipine, captopril, phenoxybenzamine, prostacyclin and indomethacin on cyclosporin A induced impairment of rat renal function.  
European Journal of Clinical Investigation, 16, 540-548.

Dieperink, H., Leyssac, P.P., Starklint, H. & Kemp, E. (1986b)  
Nephrotoxicity of cyclosporin A. A lithium clearance and micropuncture study in rats.  
Transplantation Proceedings, XV, No 4, suppl 1 & 2, 2736-2741.

Fish oil dietary supplementation in patients with Raynaud's Phenomenon: A double blind, controlled, prospective trial.
The American Journal of Medicine, 86, 158-164.

A radioimmunoassay to measure Cyclosporin A in plasma and serum samples.
Journal of Immunoassay, 2 (1), 19-32.

A radioisotope method for simultaneous determination of the glomerular filtration rate and the effective renal plasma flow.
Netherlands Journal of Medicine, 20, 97-103.

A Clinical Appraisal of the plasma concentration and endogenous clearance of creatinine.
The American Journal of Medicine, 32, 65-79.

No rise in glomerular filtration rate after protein loading in cirrhatics.
Kidney International, 32, suppl 22, S32-S34.

Duarte, C.G., Elveback, L.R. & Liedtke, R.R. (1980a)
Creatinine. In Renal Function tests. Edit. C.G. Duarte, Chapter 1
Boston, Mass: Little, Brown and Company.

Duarte, C.G., Elveback, L.R. & Liedtke, R.R. (1980b)
Glomerular Filtration Rate and Renal Plasma Flow. In Renal Function tests. Edit. C.G. Duarte, Chapter 2
Boston, Mass: Little, Brown and Company.

Du Bois, E.F. (1927)

Prostaglandins and the kidney.
Increase of glomerular filtration rate following amino-acid infusion is suppressed by indomethacin in normal subjects.

Modification of experimental nephrotoxicity with fish oil as the vehicle for cyclosporine.
Transplantation, 43, 2, 271-274.

Fish Oil Vehicle for Cyclosporine lowers Renal Thromboxanes and reduces experimental nephrotoxicity.
Transplantation Proceedings, XIX, No. 1, 1403-1406.

Cyclosporine-induced acute renal dysfunction in the rat. - Evidence of arteriolar vasoconstriction with preservation of tubular function.
Transplantation, 44, 135-141.

Long term results of renal transplantation with Cyclosporin.

Prostaglandin E₁ and Prostaglandin I₂ modulation of superoxide production by human neutrophils.
Biochemical and Biophysical Research Communications, 113, No 2, 506-512.

Cyclosporin in combination with calcium channel blockers.

Predictors of renal function in diabetic and non-diabetic renal disease.
Clinica Chimica Acta, 133, 169-175.

Does Nifedipine ameliorate Cyclosporin A nephrotoxicity?
British Medical Journal, 295, 310.

Renal vascular effects of endoperoxide analogues, prostaglandins and arachidonic acid.
N-Acetyl-B-Glucosaminidase excretion during Cyclosporin A
treatment.
Human Toxicology, 3, 416.

Analysis of Urinary Metabolites of Thromboxane and Prostacyclin by
negative-ion chemical-ionization Gas Chromatography/mass
spectrometry.
In Advances in Prostaglandin, Thromboxane and Leukotriene
York: Raven Press.

Analysis of Prostacyclin and Thromboxane Biosynthesis in
Cardiovascular Disease.
Circulation, 67, 6, 1174-1177.

The Effect of Conversion from Cyclosporine to Azathioprine
Immunosuppression for intractable nephrotoxicity.
Transplantation Proceedings, XV, No 4, suppl 1, 2869-2873.

Foegh, M.L., Zmudka, M., Cooley, C., Winchester, J.F., Helfrich, G.B.,
Urine i-TXB₂ in renal allograft rejection.
Lancet, ii, 431-434.

In vitro immunosuppressive properties of Cyclosporine metabolites.
Transplantation, 43, 1, 123-127.

Fries, D., Hiesse, C., Santelli, G., Gardin, J.P., Cantarovich, M.,
Triple therapy with low dose Cyclosporine, Azathioprine and
steroids: Long term results of a Randomized Study in Cadaver Donor
Renal Transplantation.
Transplantation Proceedings, XX, No 3, suppl 3, 130-135.

Gabriel, R. (1985)
Post Graduate Nephrology.

Garr, M.D. & Paller, M.S. (1988)
Cyclosporine augments the renal vasoconstrictor response to
norepinephrine.
Kidney International, 33, 442.

The effect of salt intake on cyclosporin-induced impairment of
renal function in rats.
Transplantation, 38, 4, 412-417.
Gilas, T. (1985)  
Prevention of Cyclosporin (CyA)-induced nephrotoxicity in rats by 16, 16 dimethyl prostaglandin E2 (dm PGE2).  

Cyclosporine Nephrotoxicity in orthotopic Liver Transplantation.  
*Transplantation Proceedings, XX*, No 3, suppl 3, 401-404.

Cyclosporine inhibits prostacyclin formation in cultured human endothelial cells.  

Grace, A.A. (1988)  
Cyclosporine A nephrotoxicity – the role of Thromboxane A2.  
*Prostaglandins, Leukotrienes and essential fatty acids, 32*, 157-164.

Cyclosporine A enhances platelet aggregation.  

Green, C. (1988)  
Recent progress in organ transplantation.  

Early and late forms of Cyclosporine Nephrotoxicity: Studies in cardiac transplant recipients.  
*American Journal of Kidney Diseases, IX*, No 1, 12-22.

Platelets in clotting. In *The Platelet in Health and Disease*.  

BM 13.177, A selective blocker of platelet and vessel wall thromboxane receptors, is active in man.  
*Lancet*, i, 991-993.

Characterization of N,N'-bis (3-Picolyl) -4-Methoxy-Isophtalamide (Picotamide)as a dual Thromboxane synthase Inhibitor/Thromboxane A2 receptor Antagonist in human platelets.  
*Thrombosis and Haemostasis, 61*, 3, 479-484.
Role of Proaggregatory and Antiaggregatory Prostaglandins in Hemostasis. (Studies with combined thromboxane synthase inhibition and thromboxane receptor antagonism.)
Journal of Clinical Investigation, 80, 1435-1445.

Bioassay of prostacyclin and endothelium-derived relaxing factor (EDRF) from porcine aortic endothelial cells.
British Journal of Pharmacology, 87, 685-694.

Australian Multicentre Trial in Cadaver Renal Transplantation. Comparison of short- and long-term Cyclosporine A therapy with conventional therapy.
Transplantation Proceedings, XIX, No 1, 1833-1834.

Binding of thromboxane A$_2$ / prostaglandin H$_2$ agonists to human platelets.

Halushka, P.V. & Lefer, A.M. (1987)
Thromboxane A$_2$ in health and disease.
Federation Proceedings, 46, No 1, 131-132.

Hamberg, M., Svensson, J. & Samuelsson, B. (1975)
Thromboxanes; A new group of biologically active compounds derived from prostaglandin endoperoxides.
Proceedings of the National Academy of Science USA., 72, 8, 2994-2998.

Long term nephrotoxicity of Cyclosporin A in Transplantation.
Dialysis and Transplantation, 11, 2, 146-160.


Decrease of Renal Blood Flow and Glomerular Filtration rate after a first oral dose of Cyclosporin in Renal Transplant patients.
Nephrology, Dialysis and Transplantation, 3, 4, 571.

Comparison between inulin and $^{51}$Cr labelled edetic acid for the measurement of glomerular filtration rate.
Lancet, ii, 1110-1112.
Herrera, J., Rodriguez-Iturbe, B., Parra, G., Coello, J., Garcia, R.,
Urinary prostaglandin E and kallikrein activity in glomerular
hyperfiltration induced by a meat meal in man.

Current concepts.
In: Cyclosporin 5 years on, Proceedings of a workshop held in

Low dose cyclosporine effective in severe psoriasis: A double
blind study.
Transplantation Proceedings, XX, No 3, Suppl 4, 32-41.

Blood cyclosporin concentrations and renal allograft dysfunction.
British Medical Journal, 293, 1057-1059.

Renal hemodynamics and limitations of creatinine clearance in
determining filtration rate in glomerular disease.

Humes, H.D., Jackson, N.M., O'Connor, R.P., Hunt, D.A. & White, M.D.
(1985)
Pathogenic mechanisms of Nephrotoxicity: Insights into
Cyclosporine Nephrotoxicity.
Transplantation Proceedings, XVII, No 4, suppl 1, 51-62.

Humphrey, P.P.A., Hallet, P., Hornby, E.J., Wallis, C.J., Collington,
Pathophysiological Actions of Thromboxane A2 and Their
Pharmacological Antagonism by Thromboxane Receptor Blockade With
GR32191.
Circulation, 81, suppl 1, I-42 - I-52.

Hunt, S.A., Stinson, E.B., Oyer, P.E.,Billingham, M.E., Gamberg, P.,
Results of "immunoconversion" from cyclosporine to Azathioprine in
heart transplant recipients with progressive nephrotoxicity.
Transplantation Proceedings, XIX, 2522-2524.

Bovine Endothelial Cells in culture produce Thromboxane aswell as
Prostacyclin.
Journal of Clinical Investigation, 67, 1292-1296.

Delayed function of cadaver kidney transplants: influence of
cyclosporine on ultimate function.
Clinical Transplantation, 1, 81-87.

Platelet and vessel associated prostacyclin and thromboxane A2/ prostaglandin endoperoxide receptors.
European Journal of Clinical Investigation, 18, 1-8.

The continuing need for Quality Assessment of Cyclosporine Measurement.
Clinical Chemistry, 35, 7, 1309-1312.

Antagonism of the Thromboxane-sensitive contractile systems of the rabbit aorta, dog saphenous vein and guinea pig trachea.

Triple therapy in cadaver renal transplantation.
British Journal of Surgery, 75, 4-8.

Cyclosporine-Induced Nephrotoxicity: Role of Prostaglandins.
Transplantation Proceedings, XIX, No 1, 1742-1744.

The Effects of Cyclosporine on human platelet aggregation and thromboxane release.
Transplantation Proceedings, XXI, No 1, 948-949.

Kahan, B.D. (1986)
Cyclosporine nephrotoxicity: Pathogenesis, prophylaxis, therapy and prognosis.
American Journal of Kidney Diseases, VIII, 323-331.

Kahan, B.D. (1985)
Individualization of cyclosporine therapy using pharmacokinetic and pharmacodynamic parameters.
Transplantation, 40, 5, 457-476.

Immunopharmacological monitoring of Cyclosporin A treated recipients of cadaveric kidney allografts.
Transplantation, 34, No 1, 36-45.

Cyclosporine and urinary prostaglandins.
Reduction of Renal Function by Newer Nonsteroidal Anti-inflammatory Drugs.
The American Journal of Medicine, 64, 804-807.

Calcium-stimulated synthesis of vasodilator renal microvascular prostanooids.
American Journal of Nephrology, 6, suppl 1, 46-48.

Interstitial fibrosis in renal allografts after 12 to 46 months of Cyclosporin treatment: beneficial effect of low doses in early post-transplantation period.
Lancet, 2, 950-953.

Prostaglandin production by homogeneous cultures of rat glomerular epithelial and mesangial cells.


Kidney Transplantation - State of the Art.
Transplantation Proceedings, XXI, No 1, 1425-1429.

Serum Creatinine and Renal Function.
Annual Review of Medicine, 39, 465-490.

Outcome of renal transplants with respect to the dose of cyclosporin.
Nephrology, Dialysis and Transplantation, 3, 4, 572.

Glomerular Prostaglandin and Thromboxane Synthesis in Rat Nephrotoxic Serum Nephritis.
Journal of Clinical Investigation, 72, 1439-1448.


Modification of the renal hemodynamic response to vasoconstrictors by calcium antagonists.
American Journal of Nephrology, 7, suppl 1, 7-16.
Effects of Cyclosporine on the isolated perfused rat kidney.
Transplantation, 43, 6, 795-799.

GR32191 A novel thromboxane receptor blocking drug: effects upon platelets and vascular and airways smooth muscle in vivo.
British Journal of Pharmacology, Proceedings supplement, 93, 43.

GR32191, a highly potent and specific thromboxane A2 receptor blocking drug on platelets and vascular and airways smooth muscle in vitro.
British Journal of Pharmacology, 97, 783-794.

Mechanism of Cyclosporin A Hypertension.
Transplantation Proceedings, XIX, No 1, 1262-1263.

Evaluation of the vascular thromboxane A2 receptor blocking activity of GR32191 in man.
British Journal of Clinical Pharmacology, 26, 662.

In vivo redirection of prostaglandin endoperoxides into 6-keto PGF1\alpha formation by thromboxane synthetase inhibitors in the rat.
Thrombosis Research, 25, 15-27.

Prevention of Cyclosporine-Induced Nephrotoxicity in Rats by 16, 16 Dimethyl Prostaglandin E2.
Transplantation Proceedings, XVII, No 1, 1381-1383.

Prevention of Cyclosporine (CyA) nephrotoxicity by synthetic prostaglandins.
Clinical Nephrology, 25, suppl 1, 89-94.

Synthesis of prostacyclin from platelet-derived endoperoxides by cultured human endothelial cells.
Journal of Clinical Investigation, 66, 979-986.

Marsh, D. & Frasier, C. (1965)
Reliability of Inulin for determining volume flow in rat renal cortical tubules.

311
Pathophysiology of Cyclosporin therapy-associated renal functional disturbance.

The Effect of Cyclosporine on Organ Blood Flow.

The effect of Nifedipine on renal function in normotensive Cyclosporin A-treated renal allograft recipients.
Nephrology, Dialysis and Transplantation, (Unpublished observations)

Cyclosporine: Five years' experience in cadaveric renal transplantation.
The New England Journal of Medicine, 310, 148-154.

Miach, P.J. (1986)
Cyclosporin A in Organ Transplantation.
The Medical Journal of Australia, 145, 146-150.


Cyclosporine Nephrotoxicity.
Advances in Nephrology, 17, 303-320.

Histology of Cyclosporin nephrotoxicity.

Morphological Findings in Kidney Transplants after Treatment with Cyclosporine.
Transplantation Proceedings, XV, No 4, suppl 1, 2821-2835.

Reversibility of cyclosporine A nephrotoxicity.
Circulation, 74, suppl II, 220.
A simple method of estimating progression of chronic renal
failure.
*Lancet*, ii, 1326-1328.

An enzyme isolated from arteries transforms prostaglandin
endoperoxides to an unstable substance that inhibits platelet
aggregation.

Prostacyclin and endothelium-derived relaxing factor: biological
interactions and significance. In *Thrombosis and Haemostasis*, Ed.
Verstraete, M., Vermylen, J., Lijnen, H.R. & Arnout, J. Ch 25, pp

Morales, J.M., Andres. A., Alcazar, J.M., Prieto, C., Diaz de Tueste,
Usefulness of fractional excretion of sodium as index of
Cyclosporine Nephrotoxicity in renal transplantation.
*Transplantation Proceedings*, XX, No 3, supp; 3, 691-699.

Morales, J.M., Andres, A., Prieto, C., Ruilope, L.M., Alcazar, J.M.,
Fractional excretion of sodium represents an index of Cyclosporine
nephrotoxicity in the early post-transplant period.
*Transplantation Proceedings*, XIX, 4005-4007.

Morris, P.J., French, M.E., Dunnill, M.S., Hunnisett, A.G.W., Ting, A.,
A Controlled trial of Cyclosporine in Renal Transplantation with
conversion to Azathioprine and Prednisolone after three months.

Intravenous cyclosporine activates afferent and efferent renal
nerves and caused sodium retention in innervated kidneys in rats.
*Proceedings of the National Academy of Science*, 82, 8222-8226.

Cyclosporine: A review of drug monitoring problems and
presentation of a simple, accurate liquid chromatographic
procedure that solves these problems.
*Clinical Biochemistry*, 19, 83-89.

Effect of Cyclosporine administration on renal hemodynamics in
conscious rats.

Estimated Creatinine Clearance by the formula of Gault and
Cockcroft in Renal Transplantation.
*Nephron*, 51, 426-427.
Myers, B.D. (1986)  
Cyclosporine nephrotoxicity.  

Cyclosporine-associated chronic nephropathy.  

The long-term course of Cyclosporine-associated chronic nephropathy.  

Cyclosporine A-Calcium Channels Interaction: A Possible Mechanism for Nephrotoxicity.  
*Transplantation Proceedings, XIX*, No 1, 1358-1362.

Cyclosporine nephrotoxicity without major organ transplantation.  
*Annals of Internal Medicine, 106*, No 3, 400-402.

Cyclosporine nephrotoxicity in spontaneously hypertensive rats.  
*Transplantation, 45*, No 4, 768-772.

Effects of Cyclosporin A on the Renin-Angiotensin system and Potassium Excretion in Renal Transplant Patients.  

Cyclosporine.  
*Surgery, Gynecology and Obstetrics, 159*, 297-308.

Prevention of delayed graft function in cadaveric kidney transplants by the Calcium Antagonist Diltiazem and the prostacyclin analogue Iloprost. Outcome of a prospective randomised clinical trial.  
*Nephrology, Dialysis and Transplantation, 3*, No 4, 572.

Glomerular thrombi in renal allografts associated with cyclosporin treatment.  
*Journal of Clinical Pathology, 38*, 253-258.
Effect of Cyclosporine on Prostacyclin Synthesis by Vascular Tissue in Rabbits.
Transplantation Proceedings, XV, No 4, 2398-2400.

Distribution and binding of cyclosporine in blood and tissues.
Transplantation Proceedings, XV, 4, suppl 1 and 2, 2419-2421.

Clinical Implications of prostaglandin and thromboxane A2 formation.
Part II 319, 12, 761-767.

Overview of physiological and pathophysiological effects of thromboxane A2.
Federation Proceedings, 46, No 1, 133-138.

Collaborative Transplant study results on immunosuppression with Cyclosporin in renal Transplantation.

Low doses of Cyclosporine, Azathioprine and Prednisolone in Renal Transplantation: Immunosuppressive Efficacy and reduced nephrotoxicity.
Transplantation Proceedings, XX, No 5, suppl 6, 26-27.

Paller, M.S. (1988)
The Prostaglandin E1 analog Misoprostol Reverses acute Cyclosporin nephrotoxicity.
Transplantation Proceedings, XX, No 3, suppl 3, 634-637.

Effects of Nva'--Cyclosporine on glomerular filtration rate and renal blood flow in the rat.
Transplantation, 43, 6, 893-895.

Paller, M.S. & Murray, B.M. (1985)
Renal Dysfunction in animal models of Cyclosporine toxicity.
Transplantation Proceedings, XVII, No 4, suppl 1, 155-159.

Paller, M.S., Murray, B.M. & Ferris, T.F. (1985)
Decreased renal blood flow after cyclosporine infusion.

The Effect of short-term glucagon infusion on kidney function in normal man.
Diabetologia, 13, 323-325.

Functional significance of Renal Prostacyclin and Thromboxane A\textsubscript{2} production in Patients with Systemic Lupus Erythematosus.
Journal of Clinical Investigation, 76, 1011-1018.

The Clinical Significance of inhibition of renal prostaglandin synthesis.

Effect of platelet-activating factor antagonist on cyclosporine nephrotoxicity.
Transplantation, 47, 592-595.

Pearson, J.D. (1989)
London: IBC Technical Services Ltd.

Functional significance of exaggerated renal thromboxane A\textsubscript{2} synthesis induced by cyclosporin A.

Effect of short-term cyclosporine administration in rats on Renin-Angiotensin and Thromboxane A\textsubscript{2}: possible relevance to the reduction in Glomerular filtration Rate.
Journal of Pharmacology and Experimental Therapeutics, 239, 229-235.


Prostaglandin and thromboxane synthesis by rat glomerular epithelial cells.
Cyclosporine-induced Nephrotoxicity: Preventive effect of a PAF-Acether Antagonist, BN 52063.
Transplantation Proceedings, XX, No 3, suppl 3, 665-669.

Reduction of Cyclosporine nephrotoxicity by prostaglandin E2 after experimental renal transplantation.
Transplantation Proceedings, XIX, 4041-4042.

Chronic Cyclosporine - Associated Nephrotoxicity.
Transplantation Proceedings, XVIII, suppl 1, 204-207.

Long term Cyclosporine for Renal Allograft Patients Does not cause Nephrotoxicity.
Transplantation Proceedings, XIX, No 1, 1761-1763.

Provoost, A.P. (1986)
Cyclosporine Nephrotoxicity in Rats with an Acute Reduction of Renal Function.

Comparative pharmacology of endothelium-derived relaxing factor, nitric oxide and prostacyclin in platelets.

Effect of Nifedipine on renal function in patients with essential hypertension.
Hypertension, 11, 5, 452-456.

Biosynthesis of thromboxane in patients with systemic sclerosis and Raynaud's phenomenon.
British Medical Journal, 292, 1037-1039.

Prostacyclin and human foetal circulation.
Prostaglandins, 18, No 3, 341-348.

Glomerular Filtration.
New England Journal of Medicine, 290, 785-792.

Changes in facial appearance during cyclosporin treatment.
Lancet, 1, 1405-1407.
Release of prostacyclin in vivo and its role in man. 
Lancet, i, 317-319.

Relationship between glomerular filtration rate and renal blood 
flow at different levels of protein-induced hyperfiltration in 
man. 
Clinical Science, 74, 11-15.

Selective Enhancement of thromboxane in macrophages and kidneys in 
Cyclosporine-induced nephrotoxicity. -dietary protection by fish 
oil. 
Transplantation, 45, 153-156.

Essential Immunology. 

The Plasma Creatinine concentration is not an accurate reflection 
of the Glomerular Filtration rate in stable renal transplant 
patients receiving Cyclosporine. 
American Journal of Kidney Diseases, X, No 2, 113-117.

Thromboxane receptor blockade attenuates the toxic effect of 
Cyclosporine in experimental renal transplantation. 
Circulation, (in press)

Rutherford, W.E., Blondin, J., Miller, J.P., Greenwalt, A.S. & Vavra, 
(1977) 
Chronic progressive renal disease: Rate of change of serum 
creatinine concentration. 

(1986) 
PGE₂ analogue reduces nephrotoxicity and immunosuppression of 
Cyclosporine in rats. 
Transplantation Proceedings, XVIII, No 4, 626-630.

Cyclosporine Nephrotoxicity. 
Toxicologic Pathology, 14, No 1, 73-82.

Volumes of distribution and clearances of intravenously injected 
creatinine in the dog. 
Liquid-chromatographic determination of Cyclosporin A in blood and plasma.  
Clinical Chemistry, 27, No 8, 1368-1371.

Reduction of nephrotoxicity and improvement of immunosuppression by combination of Cyclosporine A and Azathioprine.  
Transplantation Proceedings, XIX, 1937-1939.

Angiotensin II and eicosanoids in the control of glomerular size in the rat and human.  
American Journal of Physiology, 250, F348-F356.

Prostaglandin Synthesis by Rat Glomerular Mesangial Cells in Culture. - effects of Angiotensin II and arginine vasopressin.  
Journal of Clinical Investigation, 71, 1756-1764.

Arachidonate metabolites and the control of glomerular function.  
Federation Proceedings, 42, 14, 3058-3063.

Identification and treatment of cyclosporine-associated allograft thrombosis.  
Surgery, 100, 329-333.

Platelet-activating factor and the kidney.  
American Journal of Physiology, 251, F1-F11.

Tubuloglomerular feedback, prostaglandins and angiotensin in the autoregulation of glomerular filtration rate.  

Is Cyclosporine-induced Chronic Nephrotoxicity Inevitable?  
Transplantation Proceedings, XIX, No 1, 1745-1748.

Cyclosporine, the Renin-Angiotensin-Aldosterone System, and Renal Adverse Reactions.  
Transplantation Proceedings, XV, No 4, suppl 1, 2719-2725.

Chronic renal damage caused by Cyclosporine.  
Transplantation Proceedings, XIX, No 3, Suppl 3, 792-799.
Platelet-derived growth factor and prostacyclin in kidney transplant rejection.
Transplantation Proceedings, XIX, No 4, suppl 5, 102-107.

Smeesters, C., Chaland, P., Giroux, L., Moutquin, J.M., Etienne, P.,
Prevention of acute Cyclosporine A nephrotoxicity by a thromboxane synthetase inhibitor.
Transplantation Proceedings, XX, No 2, suppl 2, 663-669.

Smith, H.W. (1951)

Prostaglandins, 22, No 3, 353-363.

In vitro prostaglandin synthesis by various rat renal preparations.
Biochimica et Biophysica Acta, 710, 45-52.

Human glomeruli release fatty acids which stimulate thromboxane synthesis in platelets.

Stanek, B., Kovarik, J., Rasoul-Rockenschaub, S. & Silberbauer, K.
(1987)
Renin-angiotensin-aldosterone system and vasopressin in cyclosporine-treated renal allograft recipients.
Clinical Nephrology, 28, No 4, 186-189.

Cyclosporine Nephrotoxicity: studies in Laboratory Animals.
Transplantation Proceedings, XVII, No 4, suppl 1, 145-154.

Effect of Acute Cyclosporine administration on renal hemodynamics in the rat.
Human Immunology, 13, 64-65.

Treatment with Verapamil and ATP-MgCl₂ reduces Cyclosporine nephrotoxicity.

Renal Interstitial Fibrosis and vascular changes - occurrence in patients with Autoimmune Diseases treated with Cyclosporine.
Archives of Internal Medicine, 146, 2007-2010.


Cyclosporine: Immunology, Toxicity and Pharmacology in 
Experimental Animals. 

Tomlanovich, S., Golbetz, H., Perlroth, M., Stinson, E. & Myers, B.D. 
(1986) 
Limitations of creatinine in Quantifying the severity of 
Cyclosporine-induced chronic nephropathy. 
American Journal of Kidney Diseases, VIII, 5, 332-337.

Cyclosporine and sodium and potassium excretion in the rat. 
Transplantation Proceedings, XV, No 4, suppl 1, 2730-2735.

Effects of Cyclosporine on human endothelial cell cultures. 
Toxicologic Pathology, 14, 2, 210-212.

Tyler, H.M. (1983) 
Dazoxiben: a Pharmacological tool or a clinical candidate? 
British Journal of Clinical Pharmacology, 15, 135-165.

Uranga, J., Fuenzalida, R., Rapoport, A.L & del Castillo, E. (1979) 
Effect of Glucagon and Glomerulopressin on the renal function of 
the dog. 
Hormone and Metabolic Research, 11, 275-279.

Low dose cyclosporine for cadaveric renal transplantation. 
Transplantation, 45, 320-323.

Vanrenterghem, Y., Roels, L., Lerut, T., Gruwez, J., Michielsen, P., 
Gresele, P., Deckmyn, H., Colucci, M., Arnout, J. & Vermylen, J. 
(1985) 
Thromboembolic complications and haemostatic changes in 
cyclosporin treated cadaveric kidney allograft recipients. 
Lancet, i, 999-1002.

Vanrenterghem, Y., Waer, M., Roels, L., Lerut, T. & Michielsen, P. 
(1987) 
Is cyclosporine-associated nephrotoxicity progressive? 
Transplantation Proceedings, XIX, 4031-4032.

Varghese, Z., Chan, M.K., Steele, L.V., Sweny, P., Fernando, O.N. & 
How to measure Cyclosporin. 
Lancet, i, 1407-1408.
Elective conversion from cyclosporin to azathioprine: long term follow up.

Acute cellular rejection or cyclosporine A nephrotoxicity? A review of transplant renal biopsies.
American Journal of Kidney Diseases, IV, No 2, 185-191.

Verstraete, M. (1983)
Introduction: Thromboxane in Biological Systems and the possible impact of its inhibition.
British Journal of Clinical Pharmacology, 15, S7-S11.

Dihydroergotoxine (Hydergine) to prevent cyclosporine nephrotoxicity in kidney transplant recipients.
Transplantation Proceedings, XIX, No 1, 1737-1738.

Impaired fractional excretion of lithium: a very early marker of cyclosporine nephrotoxicity.
Transplantation Proceedings, XIX, 4147-4148.

Cyclosporine suppression of endothelial prostacyclin generation.
Transplantation, 45, 793-796.

Prevention of post-transplant acute tubular necrosis by the calcium channel antagonist Diltiazem: a prospective randomized study.
American Journal of Nephrology, 7, 287-291.

Influence of the calcium antagonist Diltiazem on Delayed Graft Function in Cadaveric Kidney Transplantation: Results of a 6-month follow-up.
Transplantation Proceedings, XIX, No 1, 1353-1357.


Prolonged cyclosporine therapy to induce solid engraftment after renal transplantation.
Cyclosporin A treatment for refractory Rheumatoid Arthritis.
Arthritis and Rheumatism, 30, no 1, 11-17.

Acute effects of intravenous cyclosporine on renal function in healthy humans.
Transplantation Proceedings, XXI, No 1, 915-917.

Wesson, L. (1969)
New York: Grune and Stratton.

Long-Term Effects of Cyclosporine on Renal Function in Liver Transplant recipients.
Transplantation, 43, 5, 641-647.

Cyclical changes in Cyclosporin-induced nephrotoxicity.
Human Toxicology, 3, 416.

Patterns of N-Acetyl-B-D-Glucosaminidase excretion after renal transplantation.

Whiting, P.H., Ross, I.S. & Borthwick, L. (1979)
Serum and urine N-acetyl-beta-D-glucosaminidase in diabetics on diagnosis and subsequent treatment and stable insulin-dependent diabetics.
Clinica Chimica Acta, 92, 459-463.

Lithium clearance measurements as an indication of cyclosporin A nephrotoxicity in the rat.
Clinical Science, 74, 173-178.

Pathological changes in rats receiving CyA at immunotherapeutic dosage for 7 weeks.
British Journal of Experimental Pathology, 64, 437-444.

Experimental Cyclosporin A nephrotoxicity.
British Journal of Experimental Pathology, 63, 88.

Whiting, P.H., Thomson, A.W., Cameron, I.D. & Simpson, J.G. (1980b)
Hepatic and Renal Function in rats receiving immunotherapeutic doses of cyclosporin A.
British Journal of Experimental Pathology, 61, 631-634.
Pharmacological interactions between prostacyclin and thromboxanes.  
*British Medical Bulletin, 39*, No 3, 232-238.

Measurement of True Glomerular Filtration Rate in Renal Transplant Patients Receiving Cyclosporine.  
*Transplantation Proceedings, XIX*, No 1, 1739-1741.

Plasma eicosanoids, platelet function and cold sensitivity.  
*British Journal of Surgery, 76*, 401-405.

Obstructive Nephropathy in the Rat – possible roles for the Renin-Angiotensin system, prostaglandins and thromboxanes in post obstructive renal function.  
*Journal of Clinical Investigation, 65*, 400-412.

Cyclosporin-induced endothelial cell injury.  
*Laboratory Investigation, 55*, (4), 455-462.