THE MECHANISM OF ALTERED RENAL FUNCTION FOLLOWING URETERAL OBSTRUCTION: THE ROLE OF INFILTRATING LEUKOCYTES

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ABSTRACT

THE MECHANISM OF ALTERED RENAL FUNCTION FOLLOWING URETERAL OBSTRUCTION: THE ROLE OF INFILTRATING LEUKOCYTES.
Kevin Paul Gladstone Harris

This thesis studied a rat model of acute and reversible ureteral obstruction in which there is no necrosis and in which renal functional abnormalities are transient.

A leukocyte infiltrate was found to be one of the earliest responses of the kidney to ureteral obstruction, occurring within four hours of obstruction, with the peak response occurring at 12 hours. The leukocytes form distinctive rings around the tubules, particularly distal tubules. The leukocyte cell infiltrate consists predominantly of macrophages with a lesser number of T-lymphocytes mainly of the cytotoxic, suppressor cell subclass. The kinetics of the macrophage invasion temporally correlates with both the decline in glomerular filtration rate and enhanced thromboxane B₂ excretion. In addition the infiltrate correlates with alterations in tubular function.

Total body irradiation of rats prior to the onset of obstruction so as to prevent the leukocyte infiltrate in the kidney, both reduces thromboxane B₂ excretion and significantly improves renal haemodynamics after release of 24 hours of obstruction. This implies that infiltrating leukocytes contribute to the decline in glomerular filtration rate and renal plasma flow seen after obstruction, possibly via the production of vasoactive prostanoids such as thromboxane A₂. The elimination of the leukocyte infiltrate from the obstructed kidney, however, does not return the function of the post-obstructed kidney to normal.

Glomeruli isolated from rats with ureteral obstruction demonstrate an enhanced ability to produce a variety of prostanoids, including thromboxane A₂ and this also appears to play a role in the functional changes following ureteral obstruction.

The cortex of the obstructed kidney produces a unique chemoattractant that is specific for macrophages. This substance behaves biochemically as a lipid, and may account (in part) for the macrophage infiltrate following ureteral obstruction.
TO TINA, CHARLES AND KATHERINE
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ABBREVIATIONS

ADH    Antidiuretic Hormone
BSA    Bovine Serum Albumin
BUO    Bilateral Ureteral Obstruction
BW     Body Weight
CAPD   Continuous Ambulatory Peritoneal Dialysis
CCK    Contralateral Control Kidney
Cm     Inulin Clearance
CPAH   PAH Clearance
DNA    Deoxyribonucleic Acid
EDTA   Ethylenediamine tetraacetetic acid
FITC   Fluorescein Isothiocyanate Conjugate
GFR    Glomerular Filtration Rate
HBSS   Hank's Balanced Salt Solution
Hct    Haematocrit
HPLC   High Pressure Liquid Chromatography
IgG    Immunoglobulin G
Kur    Glomerular ultrafiltration coefficient
LC     Leukocyte Common Antigen
mRNA   Messenger Ribonucleic Acid
OBS    Obstructed (kidney)
PAH    Para-aminomhippuurate
PBS    Phosphate Buffered Saline
PgE2   Prostaglandin E2
PMN    Polymorphonuclear leukocyte (Neutrophil)
POK    Post Obstructed Kidney
RBF    Renal Blood Flow
RPF    Renal Plasma Flow
sem    Standard error of the mean
SNGFR  Single Nephron GFR
TCA    Trichloroacetic acid
TLC    Thin Layer Chromatography
TxA2   Thromboxane A2
TxB2   Thromboxane B2
UOO    Unilateral Ureteral Obstruction
WCC    White Cell Count
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Although it somehow seems invidious to mention specific individuals in this regard, I feel particularly indebted to the following:

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Dr Saulo Klahr, formerly Chief of the Renal Division at Washington University St Louis and now Co-Chairman in the Department of Medicine at that institution. On my arrival in St Louis I started work in Dr Klahr's laboratory. As the world's leading authority on the pathophysiology of ureteral obstruction Dr Klahr's mentorship provided the solid basis for the research presented in this thesis. Despite his many commitments he was always able to provide support, constructive criticism and advise when required.

Dr George F Schreiner, then associate professor in the Renal Division in St Louis whose innovative approach to science provided the stimulation to believe that urinary tract obstruction might have anything to do with the immune system.
I also wish to acknowledge the following people for their assistance and technical support:

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Mrs. Sue King, Mrs. Claire Pederson and Mr. Gary Grabau for their technical assistance and Mr. Jesse Yates for his companionship and support in the laboratory whilst the experiments were being conducted.

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Finally I wish to acknowledge the patience, understanding and support provided by my wife Tina and my children Charles and Katherine during the time it took to complete this thesis.
THE FOLLOWING PUBLICATIONS CONTAIN WORK PRESENTED IN THIS THESIS


DEFINITION OF THE PROBLEM

The functional and structural changes that result from obstruction to the urinary tract are complex. The clinical consequences of obstruction to the urinary tract are relevant to both the practice of surgeons (urologists) and physicians (nephrologists). Therefore, the pathophysiology of this process has been studied by both disciplines, albeit from rather different viewpoints. As such, it is important to distinguish between the terms *obstructive uropathy*, which refers to the structural or functional changes in the urinary tract that impede the normal flow of urine, and *obstructive nephropathy*, which refers to the renal disease caused by impaired flow of urine or tubular fluid. Hydronephrosis is used to describe the dilatation of the urinary tract [Bricker NS 1971].

Obstructive uropathy is a common entity, occurring at all ages. Hydronephrosis has been found at post-mortem in 3.5 to 3.8% of patients, with equal distribution between males and females [Bell ET 1946]. Hydronephrosis is found at autopsy in about 2% of children and is due mostly to congenital anomalies of the urinary tract [Campbell MF 1970]. In middle age, kidney stones are a common cause of obstruction to the urinary tract. More than a million people in the USA have kidney stones, 80% of whom are men between the ages of 20 and 40 years [National Kidney and Urologic Diseases Advisory Board 1990]. After the age of 60 obstructive uropathy occurs more frequently in males than in females due to the increased incidence of benign prostatic hyperplasia and carcinoma of the prostate. It has been calculated that 166 patients per 100,000 population were hospitalized in the USA in 1985 with a presumptive diagnosis of obstructive uropathy [National Kidney and
Obstructive uropathy was the fourth leading diagnosis at discharge among male patients with kidney and urological disorders with a rate of 242 patients per 100,000 discharges [National Kidney and Urologic Diseases Advisory Board 1990]. In females, obstructive uropathy ranked sixth among the diagnoses at discharge in patients with kidney and urological disorders with a rate of 94 per 100,000 population. In 1985, in the USA, 387 patient visits per 100,000 patient visits were ascribed to obstructive uropathy. Some 450,000 surgical procedures are performed yearly for benign prostatic hyperplasia, making this the most common operation in males. In 1985, about 80% of these operations were performed on men age 65 or older [National Kidney and Urologic Diseases Advisory Board 1990].

Table P.1 Classification of obstructive nephropathy.

<table>
<thead>
<tr>
<th>Degree:</th>
<th>Total or complete (&quot;high grade&quot;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Partial or incomplete (&quot;low grade&quot;)</td>
</tr>
<tr>
<td>Duration:</td>
<td>Acute (hours or days); Chronic (months or years)</td>
</tr>
<tr>
<td>Location:</td>
<td>Upper urinary tract</td>
</tr>
<tr>
<td></td>
<td>Ureteropelvic junction</td>
</tr>
<tr>
<td></td>
<td>Ureter</td>
</tr>
<tr>
<td></td>
<td>Ureterovesical junction</td>
</tr>
<tr>
<td></td>
<td>Lower urinary tract</td>
</tr>
<tr>
<td></td>
<td>Bladder</td>
</tr>
<tr>
<td></td>
<td>Urethra</td>
</tr>
</tbody>
</table>

Obstructive uropathy is classified according to 1) the degree, 2) the duration, and 3) the site of the obstruction (see Table P.1). The extent or degree of the obstruction is said to be high grade when it is complete, or low grade when it is
partial or incomplete. When the obstruction is of short duration it is said to be acute and it is usually due to calculus, blood clot or sloughed papilla. Obstruction that develops slowly and is long lasting is said to be chronic, as in congenital ureteropelvic or ureterovesical abnormalities and retroperitoneal fibrosis. Upper tract obstruction is that located above the ureterovesical junction; this type of obstruction is usually unilateral in nature. Lower tract obstruction refers to lesions located below the ureterovesical junction and by definition is bilateral in nature.

Potential causes of obstruction to the urinary tract in humans are listed in Table P.2.

As a result of obstructive uropathy there may be marked alterations in renal function (obstructive nephropathy). This may be either acute with mainly functional changes, or more chronic in which case structural damage commonly results. The acute changes may recover following the effective release of the obstruction, but any structural changes will be permanent leading to a chronic loss of functioning renal tissue and thus chronic impairment of renal function. Indeed obstruction to the urinary tract remains a major cause of renal impairment world-wide. Since renal disease may progress independent of the initial insult once a critical reduction in renal mass has occurred [Walser M 1990], end stage renal failure may develop as a result of obstructive uropathy even once the obstruction is relieved. In Leicester currently 7.6% of the patients on the end-stage renal failure program (CAPD and haemodialysis) have developed renal failure at least in part as a result of obstruction to the urinary tract. These include patients with a diagnosis of chronic interstitial nephritis as a result of congenital or acquired obstructive uropathy and renal stone disease.
### Table P.2 Causes of urinary tract obstruction.

<table>
<thead>
<tr>
<th>1. Intrinsic Causes</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Intraluminal</td>
</tr>
<tr>
<td>- Intratubular deposition of crystals (uric acid, sulphates)</td>
</tr>
<tr>
<td>- Stones</td>
</tr>
<tr>
<td>- Papillary tissue</td>
</tr>
<tr>
<td>- Blood clots</td>
</tr>
<tr>
<td>B. Intramural</td>
</tr>
<tr>
<td>- Functional</td>
</tr>
<tr>
<td>- Ureter (ureteropelvic or ureterovesical dysfunction)</td>
</tr>
<tr>
<td>- Bladder (neurogenic): spinal cord defects, or trauma, diabetes, multiple sclerosis; Parkinson's disease, cerebrovascular accidents</td>
</tr>
<tr>
<td>- Bladder neck dysfunction</td>
</tr>
<tr>
<td>- Anatomic</td>
</tr>
<tr>
<td>- Tumours</td>
</tr>
<tr>
<td>- Infection-granuloma</td>
</tr>
<tr>
<td>- Strictures</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2. Extrinsic Causes</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Originating in the reproductive system</td>
</tr>
<tr>
<td>- Prostate: Benign hypertrophy or cancer</td>
</tr>
<tr>
<td>- Uterus: Pregnancy, tumours, prolapse, endometriosis</td>
</tr>
<tr>
<td>- Ovary: Abscess, tumour, cysts</td>
</tr>
<tr>
<td>B. Originating in the vascular system</td>
</tr>
<tr>
<td>- Aneurysms (aorta, iliac vessels)</td>
</tr>
<tr>
<td>- Aberrant arteries (ureteropelvic junction)</td>
</tr>
<tr>
<td>- Venous (ovarian veins, retrocaval ureter)</td>
</tr>
<tr>
<td>C. Originating in the gastrointestinal tract</td>
</tr>
<tr>
<td>- Crohn's disease, pancreatitis, appendicitis, tumours</td>
</tr>
<tr>
<td>D. Originating in the retroperitoneal space</td>
</tr>
<tr>
<td>- Inflammatory</td>
</tr>
<tr>
<td>- Fibrosis</td>
</tr>
<tr>
<td>- Tumour, haematomas</td>
</tr>
</tbody>
</table>
The purpose of this thesis is to define in detail the pathophysiologic mechanisms which result in altered renal function following high grade (complete) and acute ureteral obstruction. The major contribution made by infiltrating cells to altered renal haemodynamics will be determined with reference to the cell type involved, the kinetics of their infiltration and the factors responsible for their recruitment into the kidney following ureteral obstruction.
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1.1 Physiology of normal urine flow and micturition.

The movement of urine from the renal pelvis to the bladder depends on hydrostatic pressure, ureteral peristalsis, and the rate of urine flow [Kii F 1973, Weiss RW 1983]. Once the bladder is filled to a certain capacity, sympathetic and parasympathetic stimuli are activated, leading to relaxation of the urethral sphincter and contraction of the bladder [McGuire EJ 1983]. Although the ureterovesical junction has no discrete sphincter, the tangential manner in which the ureters enter the bladder results in their closure at this level when the intravesical pressure is increased [Tanagho EA 1963, Woodburne RT 1964]. Thus, low intraureteral pressures are maintained during micturition. Obviously, obstruction or limitation to the flow of urine at any point in the urinary system will impede the normal transit of the urine from the kidney to the exterior. Impairment to urine flow will increase intraluminal pressure and may cause parenchymal damage through a series of direct and indirect effects. Similar changes may occur if ureteral peristalsis is ineffective or if ureteral closure at the ureterovesical junction does not occur at the time of micturition (reflux nephropathy).

1.1.1 Normal ureteral function.

Urine transfer from the calices and pelvis into the ureter is initiated by electrical activity generated by pacemaker sites located in the region of the calices. This electrical activity spreads distally and gives rise to pelvi-ureteral contraction and peristalsis which propel the urine from the kidney to the bladder. At low or normal urine flows propagation of the electrical activity initiated in the calyces is usually
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blocked at the renal pelvis or at the ureteropelvic junction resulting in greater
frequency of calyceal contractions than ureteric contractions. With increases in urine
flow ureteric and calyceal contractions occur at the same frequency. The ureteral
smooth muscle cells are the main anatomical and functional unit of the ureter. These
cells have a double layer cell membrane. The inner membrane surrounds the entire
cytosol, whereas the outer membrane surrounds the periphery of the cell except at
areas of close cell to cell contact referred to as intermediate junctions. These
junctions provide low resistance pathways for the conduction of electrical signals from
one muscle cell to another. As the renal pelvis fills with urine, pelvic pressure rises
and urine is extruded into the ureter. Since the ureter is collapsed prior to urine
evacuation from the pelvis, a bolus of urine forms in the most proximal portion of the
ureter. Contraction of the proximal ureter moves the bolus of urine in a distal
direction [Kii F 1973, Weiss RW 1983]. For efficient propulsion of the urine bolus
the peristaltic contraction must completely collapse the ureteral walls (coaptation).
Resting or basal ureteral pressures are approximately 0-5 cm of water and peristaltic
contraction waves of 20-60 cm of water are superimposed on this baseline pressure
at intervals of approximately 10-30 seconds. The urine enters the bladder across the
ureterovesical junction which under normal conditions permits only unidirectional
urine transport [Tanagho EA 1963, Woodburne RT 1964]. Intraureteral pressure at
the ureterovesical junction must exceed intravesical pressure for urine to move
unimpeded from the ureter to the bladder.

When ureteral transport of urine becomes inadequate, urine stasis occurs with
subsequent dilatation of the ureter and finally cessation of peristaltic activity. In this
setting, urine may flow from the pelvis to the bladder as a continuous column of fluid.
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The ureter can only efficiently transport a certain amount of fluid per unit time. Inadequate transport of fluid may result from either too much fluid entering the system or too little fluid exiting the ureter per unit time. Thus, ureteral dilatation and impaired peristaltic activity may result from obstruction to outflow or excessive urine flow into the system [Biancani P 1982].

The syncytial smooth muscle cells of the ureter lack defined neuromuscular junctions and depend on the diffuse release of transmitter from a bundle of nerves with subsequent spread of the excitation from one muscle to another. Available information indicates that although the autonomic nervous system is not required for peristalsis it can modulate ureteral function [Kiil F 1973, Weiss RW 1983]. The ureter has both alpha and beta adrenergic receptors. Alpha-adrenergic receptors are more abundant and appear to be excitatory whereas beta receptors are inhibitory. Various alpha-adrenergic agonists increase the force and frequency of ureteral contraction. On the other hand, beta-adrenergic agonists decrease contractile force and relaxation.

1.1.2 The bladder and control of micturition.

The bladder is part of the lower urinary tract and has the ability to store urine and to contract and expel its contents [McGuire EJ 1983]. During the storage phase, urine is kept within the bladder by means of a sphincter mechanism [Elbadawi A 1982, Gosling J 1979] which consists of 1) the bladder neck; 2) the striated and smooth muscles intrinsic to the urethra; and 3) the skeletal muscles of the pelvic floor extrinsic to the urethra (elevator ani or extrinsic rhabdomyosphincter). During filling of the normal bladder, sympathetic impulses and the elastic properties of the bladder wall prevent a rise in intravesical pressure and maintain a relatively low intravesical
pressure which facilitates urine transport across the ureterovesical junction and prevents ureteral dilatation [McGuire EJ 1983]. Under pathological conditions such as a fibrotic bladder and in some forms of neurogenic vesical dysfunction, relatively small increases in volume may result in large increases in intravesical pressure and inadequate ureteral emptying. The ureter usually decompensates when intravesical pressure approaches 40 cm of water. The urethra provides continence by generating a pressure higher than that within the bladder [[Elbadawi A 1982, Gosling J 1979, McGuire EJ 1983].

Innervation of the lower urinary tract is via sympathetic and parasympathetic efferents from the spinal cord segments S2 to S4. Afferent fibres from the lower urinary tract are carried via the pudendal, pelvic and hypogastric nerves. Progressive bladder distention increases activity of afferent fibres that travel via sympathetic nerves to the lateral columns of the spinal cord but these signals are inhibited by the parasympathetic system until a critical volume is reached when a desire to void is appreciated. Voiding is then initiated by a combination of voluntary relaxation of the extrinsic rhabdomyosphencter (bladder neck) and a fall in intraurethral pressure followed by contraction of the detrusor muscle [Elbadawi A 1982, McGuire EJ 1983]. Intravesical pressure rises and urine flow continues until the bladder is empty.
1.2 The consequences of ureteral obstruction.

Ureteral obstruction results in changes to both ureteral function and renal function.

1.2.1 Ureteral function.

The effects of acute ureteral occlusion on the dynamics of the ureter have been examined in dogs [Rose JG 1973]. Baseline pressures average 7.4 mm Hg and rise to a peak pressure of approximately 17 mm Hg during coaptation. These changes in pressure are accompanied by increases in ureteral wall tension. Three minutes after obstruction, baseline and peak values for both ureteral pressure and wall tension increase nearly twofold. Five to 20 minutes after obstruction, the baseline wall tension increases further but peak pressures change less dramatically. Thus baseline ureteral wall tension and pressure approaches peak values. One hour after obstruction, mean wall tension values at baseline and peak are threefold greater than under normal conditions but do not differ significantly from each other. Similarly, baseline ureteral pressure is threefold greater than measurements obtained in controls but does not change during contraction. At this point, coaptation does not occur and pressures generated by ureteral wall tension are transmitted directly to the renal pelvis and parenchyma. Any further increase in pressure will result in ureteral dilatation. These changes are summarised diagrammatically in Figure 1.1.
Figure 1.1  Relation of ureteral pressure and ureteral wall tension at various time periods following acute ureteral obstruction in dogs. Changes are shown as fold increase over baseline at time 0 (prior to obstruction). [Adapted from data in Rose GR 1973].
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In chronic ureteral obstruction baseline wall tensions are high and not significantly different from peak measurements [Rose JG 1973]. On the other hand, baseline and peak values for ureteral pressure are not significantly different from control values. This is the result of the relation between pressure and tension as expressed in Laplace’s Law: \( P = K \left( \frac{T}{R} \right) \), where \( P \) is the transluminal pressure; \( K \) is a constant; \( T \) is the wall tension, and \( R \) is the radius of the ureter. Thus in chronic ureteral obstruction normal ureteral pressures are maintained at the expense of increasing radius of the ureter. Based on these results it has been postulated that the major damage to renal tissue, which is the direct consequence of increased pressure, occurs soon after an interval narrowing of the ureteral lumen. The clinical observations that support this conclusion are as follows. 1) The highest measured ureteral pressures (40-50 mm Hg) occur during acute obstruction as seen during the passage of stones [Gillenwater JY 1978, Whitaker RH 1976]. 2) In patients with incomplete obstruction there is an inverse correlation between intrapelvic pressures and time [Michaelson G 1974]. 3) Patients with obstructive uropathy due to congenital anomalies, which tend to be static, may have relatively well preserved renal function in the fourth and fifth decades of life [Gillenwater JY 1975, Michaelson G 1974, Whitaker RH 1976].

1.2.2 Renal function

It appears that changes in the function of the obstructed kidney occur as a result of changes induced by the direct transmission of ureteral pressure to the renal substance. Thus, manoeuvres which potentiate the acute increase ureteral pressure, such as increases in urine flow (e.g. when fluid intake is increased or after the administration of diuretics) and/or augmentation of the degree of obstruction would
be expected to result in more marked changes in function. In support of this, fluid loading rats in the period following unilateral ureteral obstruction results in both a higher ureteric pressure and a lower inulin clearance in the obstructed kidney [Osborn D 1974].

The effects of complete ureteral occlusion on intratubular pressure, glomerular filtration rate and renal blood flow are summarised in Table 1.1. The effects are related to the duration and degree of obstruction. Changes in intra-tubular pressure [Gottschalk CW 1956] after ureteral occlusion depend on urine flow. During hydropaenia pressures do not change, but when urine flow is increased, as occurs during solute diuresis or expansion of the extracellular fluid volume, the intra-tubular pressure may exceed 50 mm Hg and approximate intra-glomerular pressure. These initial high pressures diminish gradually over the ensuing 24 hours [Wright FS 1982]. Beyond 24 hours, whether intra-tubular pressures remain elevated is dependent on two factors: 1) alterations in the volume status of the animal and thus flow through the tubule, and 2) whether the obstruction is bilateral or unilateral, since unilateral ureteral occlusion has less profound effects on tubular pressure than bilateral obstruction.
Table 1.1 Renal Responses to Complete Ureteral Obstruction.

<table>
<thead>
<tr>
<th></th>
<th>Ureteral Pressure</th>
<th>Proximal Tubule Pressure</th>
<th>GFR</th>
<th>RBF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilateral</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 hour</td>
<td>↑↑</td>
<td>↑300%</td>
<td>↓</td>
<td>?</td>
</tr>
<tr>
<td>24 hours</td>
<td>↑</td>
<td>↑100%</td>
<td>↓</td>
<td>↓30%</td>
</tr>
<tr>
<td>Unilateral</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 hour</td>
<td>↑↑</td>
<td>↑100%</td>
<td>↓</td>
<td>↑30%</td>
</tr>
<tr>
<td>24 hours</td>
<td>↑</td>
<td>↓30%</td>
<td>↓</td>
<td>↓40%</td>
</tr>
</tbody>
</table>

1.3 Pathophysiology of obstructive nephropathy: alterations in glomerular haemodynamics.

The effects of urinary tract obstruction on the kidney are a result of a variety of factors with complex interactions, resulting in alterations in both glomerular haemodynamics and tubular function [Klahr S 1988]. Most of the information regarding these changes is derived from studies in experimental animals using clearance and micropuncture techniques, since it is usually impossible both to define the exact time of onset of obstruction in humans and to make comprehensive functional measurements. In addition, methods used to measure renal blood flow in humans are indirect and are affected by declining renal tubular function. However, in experimental animals the relation between duration of obstruction and the changes in GFR and renal blood flow is clear.

Most experimental studies have examined the effects of complete short-term or acute ureteral obstruction (less than 36 hours duration) on renal function. Both
bilateral ureteral obstruction and unilateral ureteral obstruction have been studied, and it is apparent that there are significant differences between these two models. The effects of long standing urinary tract obstruction and partial urinary tract obstruction have been less comprehensively studied.

1.3.1 Changes in glomerular filtration following acute ureteral obstruction.

Glomerular filtration rate declines progressively following the onset of complete ureteral obstruction [Harris RH 1981]. The maintenance of glomerular filtration following ureteral obstruction is a result of i) the reabsorption of salt and water along the nephron, ii) the ability of the renal tract to dilate, and iii) alterations in renal haemodynamics.

There are four principal determinants of glomerular filtration: a) The mean hydraulic pressure gradient between the glomerular capillary lumen and Bowman's space (AP); b) Renal plasma flow (Q); c) The ultrafiltration coefficient of the glomerular capillary wall (Kf); and d) The mean oncotic pressure difference across the glomerular wall (Aπ). The way in which ureteral obstruction affects these determinants of glomerular filtration depends on the duration of the obstruction, the hydration state of the animal, and whether there is a contralateral functioning kidney.

a. Changes in the hydraulic pressure gradient.

Changes in ureteral pressure appear to be instantaneously reflected in changes in proximal tubular pressure, the latter always being higher than the former [Gottschalk CW 1956]. The extent of rise of proximal tubular pressure depends on the degree of hydration of the animal, being greatest when the animals are saline loaded, and whether one or both kidneys are obstructed. Nevertheless, independent of volume status within an hour of ureteral obstruction intra-tubular pressure has risen
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[Wright FS 1982]. At the same time glomerular capillary hydraulic pressure increases as assessed by stop flow pressure [Safirstein R 1975], but this is not proportional to the increase in intra-tubular pressure and there is a net decrease in the hydraulic pressure gradient across glomerular capillaries resulting in a decline in glomerular filtration rate. This may be the major factor responsible for the decline in glomerular filtration rate in the initial phase of ureteral obstruction. Within 5 hours of ureteral obstruction proximal tubular pressure declines. In unilateral ureteral obstruction after 24 hours of obstruction intra-tubular pressures are lower than or nearly equal to values prior to obstruction, but this does not result in an effective filtration pressure, since intra-glomerular capillary hydraulic pressure declines at an even faster rate, and falls below pre-obstruction levels. Following bilateral ureteral obstruction proximal tubular pressures are initially two-fold higher than with unilateral ureteral obstruction. By 24 hours the levels have fallen, but not back to baseline. At this time glomerular capillary pressure is not different from pre-obstruction values. Thus in this setting high intra-tubular pressures do contribute significantly to the decrease in glomerular filtration rate at this time point. These changes are summarised in Figure 1.2.
Figure 1.2  Pressure in proximal renal tubules (Pt) and proximal tubule stop-flow pressure (Psf) (a reflection of glomerular capillary pressure), before and during complete obstruction of one ureter (UUO) or both ureters (BUO). As explained in the text the pattern of the changes in these parameters depends on whether UUO or BUO is studied. However trans glomerular capillary gradient ($\Delta P = Psf - Pt$) (■) falls in both. [Adapted from Wright FS 1982].

b. Changes in renal plasma flow.

The temporal relationships between the onset of ureteral obstruction and alterations in renal plasma flow in the dog has three distinct phases [Moody TE 1975]. Similar events have been described in both the cat and rat.

In the first phase renal blood flow rises above control levels and there is a decline in renal vascular resistance, principally at the site of the afferent arteriolar vessel [Dal Canton A 1977, Moody TE 1975]. This "hyperaemic" response appears
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to be the result of an intrarenal mechanism since it occurs in both denervated [Vaughan ED Jr 1971a] and the isolated perfused kidney [Navar LG 1970]. The increase in renal blood flow and afferent arteriolar dilatation results in an increase in glomerular capillary pressure. This response maintains glomerular filtration at approximately 80% of pre-obstruction values despite a marked increase of proximal tubular pressure. The mechanism for this response involves a signal generated at a single nephron level since a wax block placed in the proximal tubule generates an identical glomerular haemodynamic response [Tanner GA 1979]. Tanner suggested that the decrease in afferent arteriolar resistance was as a result of tubuloglomerular feedback as a consequence of acutely interrupting distal delivery of tubular fluid to the macula densa. Ichikawa, however, showed that glomerular blood flow does not increase if proximal tubular pressure is maintained in the normal range in the face of tubule blockade, suggesting that the altered glomerular haemodynamics is a result of intra-tubular dynamics rather than cessation of distal delivery of tubule fluid [Ichikawa I 1982]. The increase in renal blood flow that occurs acutely in response to ureteral obstruction can be prevented by the administration of indomethacin [Allen JT 1978, Blackshear JL 1978]. This would suggest that it is due to the generation of vasodilatory prostaglandins, and indeed prostaglandin E₂ excretion does increase in response to an increase in ureteral pressure [Olsen UB 1976]. At this time the renal vascular bed is resistant to vasoconstriction induced by electrical stimulation of renal nerves, or infusion of catecholamines [Schramm 1975], and autoregulation of renal blood flow is impaired [Navar LG 1970], again suggesting a prominent vasodilating influence following ureteral obstruction.

In the second phase, approximately two to five hours after obstruction renal
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blood flow begins to decline, while ureteral pressure continues to increase. This may
be partially a consequence of augmented renal resistance, which is a direct effect of
increased ureteral pressure on the interstitium.

In the third phase ureteral pressure starts to fall towards control values and
renal plasma flow continues to decline, reaching about 30 to 50% of control values
by 24 hours. Both micropuncture studies [Dal Canton A 1979] and studies with
microspheres [Gaudio KM 1980] have shown that this vasoconstrictive response of
the kidney in response to unilateral ureteral obstruction results predominantly from an
increase in resistance of afferent arterioles. The increase in afferent arteriolar
resistance and subsequent decline in single nephron glomerular filtration rate after
ureteral obstruction can be reproduced by blockade of individual nephrons in a non-
obstructed kidney, indicating that the changes in renal haemodynamics seen at this
time interval are mediated through a control mechanism functioning at the level of the
nephron.

Following bilateral ureteral obstruction the early changes in renal
haemodynamics appear to be similar to those seen following unilateral ureteral
obstruction in that there is an initial hyperaemic phase which is blocked by
indomethacin [Allen JT 1978, Blackshear JL 1978], and the decline in glomerular
filtration rate is thus secondary to a rise in intra-tubular pressure. Renal plasma flow
falls progressively and is similar at 24 hours to that seen after unilateral ureteral
obstruction, although afferent arteriolar resistance may not increase as much. As a
result of the persistently high proximal tubular pressure and decline in renal plasma
flow it would be expected that the fall in glomerular filtration will be greater
following bilateral ureteral obstruction than unilateral ureteral obstruction. However,
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this does not occur and may reflect the presence of a greater intra-glomerular capillary pressure in animals with bilateral obstruction than in those with unilateral obstruction and also the fact that after 24 hours of obstruction the number of filtering nephrons is greater prior to and after release of obstruction in rats with bilateral ureteral obstruction than in those with unilateral ureteral obstruction [Buerkert J 1983].

The relative changes with time in ureteral pressure, renal blood flow and glomerular filtration rate are shown in Figure 1.3

Figure 1.3 The triphasic relation between renal blood flow (RBF), glomerular filtration rate (GFR) and ureteral pressure during ureteral occlusion. The three phases are designated by roman numerals and divided by vertical dashed lines. In phase I, the renal blood flow and ureteral pressure rise together. In phase II, the renal blood flow begins to decline while the ureteral pressure remains elevated and, in fact, continues to rise. Phase III shows the renal blood flow and ureteral pressure declining together. Glomerular filtration rate falls from the time of ureteral obstruction. [Moody TE 1975, Harris RH 1981].

-20-
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c. **Alteration in the ultrafiltration coefficient.**

It is clear that following ureteral obstruction glomerular filtration rate decreases to a greater extent than renal plasma flow [Harris RH 1974, Yarger WE 1974]. This results in a decreased filtration fraction in the post-obstructed kidney. This may reflect preferential constriction of the preglomerular blood vessels, as this would lower both blood flow and glomerular capillary pressure, thus resulting in a greater decrement in glomerular filtration rate than blood flow. Alternatively it suggests that there is either diversion of blood to non-filtering areas of the kidney or that there is a reduced area available for filtration per glomerulus. That the latter occurs is suggested by studies in which $K_t$ values from rats with ureteral obstruction were lower than those typically obtained in normal rats [Ichikawa I 1985].

d. **Alterations in net oncotic pressure.**

There is no information on whether changes in the oncotic pressure difference across the glomerular wall modify glomerular haemodynamics following ureteral obstruction.

1.3.2 **Effects of prolonged complete or partial ureteral obstruction on glomerular filtration and renal plasma flow.**

Following ureteral obstruction in the rat glomerular filtration rate reaches 2% of control values by 48 hours and remains at this low level. Renal plasma flow also declines, but less so [Provoost AP 1981]. Similar observations have been made in the dog [Vaughan ED Jr 1971b]. The effects of chronic partial ureteric obstruction depend on both the degree and duration of obstruction. Whole kidney glomerular filtration is reduced to one-third of control values 2 to 4 weeks following partial ureteral obstruction in the rat. Single nephron glomerular filtration rate, however, was
only reduced by 20% of control levels, suggesting that the decline in whole kidney
function was a result of a loss in the number of functioning nephrons not accessible
to micropuncture, i.e., juxtamedullary nephrons [Wilson DR 1972].

Following partial obstruction of 2 to 4 weeks duration there is a 30% decrease
in the ultrafiltration coefficient but single nephron plasma flow and glomerular
filtration is maintained near normal as a result of an increase in glomerular capillary
pressure, secondary to a decrease in afferent and efferent arteriolar resistances. This
dilatation is prostaglandin-mediated since indomethacin increases both afferent and
efferent arteriolar resistances and causes a decline in single nephron glomerular
filtration [Ichikawa I 1979].

1.3.3 The role of vasoactive hormones in post-obstructive renal haemodynamics

Two major vasoconstrictors, namely angiotensin II and thromboxane A₂, play
a major role in the decrease in renal plasma flow per nephron and the decline in
single nephron glomerular filtration rate that is seen following ureteral obstruction
[Klahr S 1988]. As well as being potent vasoconstrictors, both angiotensin II
[Ausiello DA 1984] and thromboxane A₂ [Mené P 1986] are able to contract
mesangial cells in culture and therefore can potentially reduce the glomerular capillary
area available for filtration. Figure 1.4 summarizes the effects of these two hormones
on renal function. The central and critical role of these two vasoconstrictors in
modulating post-obstructive renal haemodynamics is illustrated by the fact that if rats
are pretreated with angiotensin converting enzyme inhibitors and thromboxane
synthase inhibitors, the decline in renal function that is seen following ureteral
obstruction is virtually prevented [Purkerson ML 1989a].
THE DECREASED GFR IN OBSTRUCTION IS DUE TO

\[ \text{1. A DECREASE IN SNGFR} \]

1. DECREASE IN NET FILTRATION PRESSURE
2. DECREASE IN PLASMA FLOW PER NEPHRON \((Q_A)\)
3. DECREASE IN \(K_f\)

2. A DECREASE IN THE TOTAL NUMBER OF FILTERING NEPHRONS

\[ \text{THROMBOXANE A}_2 \ (iQ_A, iK_f, i\Delta P) \]

\[ \text{ANGIOTENSIN II} \ (iQ_A, iK_f, i\Delta P) \]

Figure 1.4  
Diagram to show the potential mechanisms where by glomerular filtration rate (GFR) may be reduced in obstructive nephropathy. GFR is reduced due to a reduction in both single nephron GFR (SNGFR) and in the total number of filtering nephrons. Initially (3-6 hours after the onset of obstruction) SNGFR falls due to reduced net filtration pressure \((\Delta P)\) as a consequence of a marked elevation in hydrostatic pressure \((P_T)\) in Bowman's space without a comparable increase in intra-glomerular pressure \((P_{ge})\). After 24 hours of obstruction the decrease in SNGFR is due mainly to a fall in intra-glomerular hydrostatic pressure \((P_{ge})\). This decrease in \(P_{ge}\) may be related to decreased plasma flow per nephron \((Q_A)\) as a consequence of increased intrarenal levels of thromboxane \(A_2\) and angiotensin II. Inhibition of thromboxane synthesis in rats with ureteral obstruction increases \(Q_A\), decreases afferent \((R_A)\) and efferent \((R_E)\) arteriolar resistances and increases the ultrafiltration coefficient \((K_f)\) suggesting that this vasoconstrictor decreases \(Q_A\) and \(K_f\) and increases \(R_A\) and \(R_E\). Angiotensin administration in normal rats has been shown to decrease \(Q_A\) and \(K_f\) and increase net filtration pressure \((\Delta P)\), presumably due to preferential constriction of the efferent arteriole. Both thromboxane \(A_2\) and angiotensin \(A_2\) may decrease \(K_f\) by contraction of mesangial cells, and hence a decrease in the total glomerular surface area available for filtration.
Ureteral obstruction results in an increase in renin secretion into the renal vein [Kaloyanides GJ 1973, Vaughan ED Jr 1971a]. This enhanced renin release could result from the stimulation of intrarenal mechanisms which may be activated by reduced delivery of sodium and chloride to the distal nephron (macula densa mechanism) or by a reduction in transmural pressure at the baroreceptor as a consequence of the dilatation of the afferent arteriole that has been previously described. The increase in release of renin is almost completely abolished by pretreatment with the cyclo-oxygenase inhibitors indomethacin or meclofenamate [Blackshear JL 1978, Cadnapaphornchai P 1978]. It is thus likely that the formation of vasodilatory prostaglandins such as prostacyclin or prostaglandin E₂ represents a necessary step for the release of renin from juxtaglomerular cells [Gerber JG 1981]. The stimulatory effect of increased ureteral pressure on renin release cannot be inhibited completely by infusion of 0.9% saline. Furthermore, the maximum renin release coincided with complete arteriolar dilatation [Eide I 1977]. These observations are compatible with the conclusion that arteriolar dilatation is the predominant stimulus to renin secretion during ureteral constriction. In addition, the generation of renal cortical prostaglandins may act as a direct stimulus to the release of renin, since prostaglandins are able to release renin from renal cortical slices [Jackson EK 1981].

Further evidence that the generation of angiotensin II plays a central role in the modulation of haemodynamic changes following ureteral obstruction is provided by the effects of angiotensin converting enzyme inhibitors and angiotensin II antagonists on post-obstructive renal function. The angiotensin II antagonist Saralasin has been shown to ameliorate the vasoconstriction that is seen in response to ureteral obstruction in rats [Yarger WE 1980], although other authors have found it ineffective
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in dogs [Moody TE 1977]. Similarly, the angiotensin converting enzyme inhibitors Captopril [Ichikawa I 1985, Yarger WE 1980] and Enalapril appear to be highly effective in ameliorating the decline in glomerular filtration rate and renal plasma flow in response to ureteral obstruction, and this effect is especially marked if the inhibitor is administered prior to the onset of obstruction [Purkerson ML 1989a]. Since converting enzyme inhibitors also block kinin activation, their effect could be secondary to enhancing the levels of vasodilator kinins, rather than inhibiting the production of the vasoconstrictor angiotensin. However, neither aprotinin, a kallikrein inhibitor that blocks kinin production, nor carboxypeptidase, an enzyme that increases kinin destruction, blocks the beneficial effect of captopril on post-obstructive renal function [Yarger WE 1980]. Thus the beneficial effect of converting enzyme inhibitors appears to be due to inhibition of the generation of angiotensin II.

The role (if any) of the kallikrein-kinin system in the modulation of renal haemodynamics following ureteral obstruction remains to be defined.

It is clear that following the onset of ureteral obstruction the hydronephrotic kidney exhibits an enhanced ability to generate thromboxane A₂ [Morrison AR 1977, Morrison AR 1978]. This prostanoid acts as a potent vasoconstrictor in the hydronephrotic kidney [Kawasaki A 1982]. That the generation of this prostanoid is pathophysiologically significant is suggested by the observations that inhibitors of thromboxane synthesis partially reverse the decline in renal function seen after obstruction [Ichikawa I 1985, Klotman PE 1986, Yarger WE 1980]. Again, prior administration of the thromboxane synthase inhibitor appears to convey more benefit [Purkerson ML 1989a]. Interestingly, the generation of thromboxane in response to ureteral obstruction is conditioned by the diet the animals are taking prior to the onset...
of ureteral obstruction. Animals fed a low protein diet do not generate as much thromboxane and exhibit less vasoconstriction in response to ureteral obstruction than animals fed a high protein diet [Ichikawa I 1985]. Furthermore, thromboxane synthase inhibitors are not effective in reversing the post-obstructive vasoconstriction in animals fed a low protein diet.

Although most experimental studies support the suggestion that thromboxane generation is important in the alteration in renal haemodynamics following ureteral obstruction this has not universally been found to be the case [Loo MH 1987, Strand JC 1981] and the reason for this discrepancy is unclear.

As described previously the haemodynamic response to ureteral obstruction varies according to whether the obstruction is bilateral or unilateral. The mechanisms responsible for these differences in renal haemodynamics are not well understood. A possible contributing factor to the observed differences is that plasma levels of atrial peptide are higher in animals with bilateral ureteral obstruction than in those with unilateral ureteral obstruction [Fried TA 1986a, Purkerson ML 1989b]. Atrial peptide causes pre-glomerular vasodilatation and post-glomerular vasoconstriction [Marin-Grez M 1986], and has been demonstrated to increase \( K_f \) in the isolated perfused glomerulus preparation [Fried TA 1986b]. In addition, the administration of exogenous atrial peptide is able to increase glomerular filtration rate following both unilateral and bilateral ureteral obstruction [Purkerson ML 1989a, Purkerson 1989b].

1.3.4 Recovery of glomerular function following ureteral obstruction.

The extent to which glomerular function recovers following the release of ureteral obstruction depends on the duration of the obstruction. This has been studied in both the dog and the rat. In the dog serial studies of glomerular filtration have
been made after one week of complete ureteral obstruction [Kerr WS Jr 1954]. Assuming the glomerular filtration rate for each kidney was half that of the whole animal prior to ureteric ligation, immediately after release of the obstruction glomerular filtration rate averaged 25% of ipsilateral control values and 16% of the concurrent values for the contralateral kidney, the latter having undergone a compensatory increase in function. Follow-up studies revealed an increase in the glomerular filtration rate of the obstructed kidney and a decline in the normal kidney, stabilizing at 2 months. In no case was there complete functional recovery of the obstructed kidney and at 2 years the glomerular filtration rate of the experimental kidney remained 50% below the simultaneous value obtained for the contralateral kidney. These changes in glomerular filtration rate were paralleled by the alterations in effective renal plasma flow, as assessed by the clearance of paraaminohippuric acid, so filtration fraction did not change. In the rat if ureteral obstruction is present for more than 72 hours a permanent decrease in glomerular filtration rate occurs [Provoost AP 1981]. Recovery of glomerular filtration rate is complete 7-9 days after release of shorter periods (less than 30 hours) of obstruction [McDougal WS 1972]. Although this would suggest that short term obstruction is completely reversible and that most of the decrease in glomerular filtration rate is functional, there is indirect evidence to suggest that the "normalization" in filtration rate may not be a consequence of a homogenous recovery in the SNGFR of all nephrons. Early studies reported that calculations of whole kidney glomerular filtration rate based on determinations of surface nephron SNGFR, were greater than those obtained from clearance measurements [Jaenike JR 1970]. This may be explained by the fact that, using Hansen's technique, 40% of superficial nephrons (those accessible to micropuncture)
were found to be filtering immediately following release of the obstruction, whereas only 12% of juxtamedullary nephrons were filtering, suggesting a selective loss of juxtamedullary nephrons [Harris RH 1974]. In addition, 3-6 hours after release of unilateral ureteral obstruction of 24 hours duration glomerular filtration values are one-sixth of those observed prior to ligation of the ureter. With time there is an increase in the glomerular filtration rate, such that by 14 and 60 days after the release of obstruction values in the post-obstructed kidney are comparable to those in the contralateral untouched kidney. However, when SNGFR and the number of filtering nephrons were determined using a modification of Hansen’s technique a decrease in the total number of filtering nephrons was found in the post-obstructed kidney, such that only 85% of the nephrons in the post-obstructed kidney were filtering, compared to 100% in the contralateral kidney [Bander SJ 1985]. The normalization of whole kidney glomerular filtration rate was therefore at the expense of hyperfiltration (increase in SNGFR) in the remaining functional nephrons, and there was a permanent decrement in the total number of functional nephrons. The mechanism responsible for this permanent loss of nephrons following ureteral obstruction remains to be defined, as does its long-term significance in terms of the development of chronic renal failure following obstructive uropathy.

1.4 Pathophysiology of obstructive nephropathy: alterations in tubular function.

Abnormalities in tubular function are common in urinary tract obstruction [Yarger WE 1972], and include changes in the renal handling of a variety of electrolytes as well as alterations in the regulation of water excretion. There is a decreased ability to concentrate the urine, and the reabsorption of sodium and other
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solutes such as phosphorus, magnesium and calcium ions is altered, as is the secretion of hydrogen and potassium. The mechanism for these alterations in function which results in the syndrome commonly referred to as "post-obstructive diuresis", remains unclear, but most of the evidence points to the major site of damage being in the distal nephron. With protracted periods of obstruction there are morphological changes in the tubules and thus, the resultant loss of function is not surprising, but even with short periods of obstruction, when there is minimal histological change, the alterations in tubular function are marked. The degree and nature of the tubular defects following obstruction depend in part on whether the obstruction is bilateral or unilateral.

1.4.1 Sodium and water reabsorption.

Unilateral ureteral obstruction. Following release of 24 hours of unilateral ureteral obstruction the quantity of sodium excreted by the post-obstructed kidney has usually been found to be similar to that excreted by the control kidney [Buerkert J 1976, Harris RH 1975], although it has also been reported to be slightly increased or decreased according to the experimental conditions employed. However, since the glomerular filtration rate is reduced to about 20% of control values following 24 hours of obstruction the fractional excretion of sodium has universally been found to be elevated in the post-obstructed kidney.

Micropuncture studies have indicated that despite the decline in SNGFR and hence the filtered load, the fraction of sodium and water reabsorbed along the proximal tubule of superficial nephrons may be increased following release of unilateral ureteral obstruction [Buerkert J 1976, Buerkert J 1978] (in contrast to release of bilateral ureteral obstruction - see below). However, reabsorption by
proximal tubules of deep nephrons is decreased [Buerkert J 1978], and this may represent one of the major sites of decreased sodium and water reabsorption following unilateral ureteral obstruction. In addition there is an increase in the fraction of filtered water delivered to the early distal tubule [Buerkert J 1976], suggesting that there is a decreased ability of the thick ascending limb of Henle's loop to resorb salt. This would in turn result in a fall in medullary tonicity. Reabsorption of sodium from the distal tubule of surface nephrons is also increased.

Thus, much of the sodium and water that appears in the urine following release of unilateral ureteral obstruction must be due to alterations in either the medullary collecting duct or in deep nephron function or both.

Changes in effective intravascular volume affect both the absolute and fractional excretion of sodium after release of unilateral ureteral obstruction. Thus, absolute sodium excretion will be reduced in volume depleted and anaesthetized rats [Harris RH 1974], when compared to awake rats [Harris RH 1975], though fractional excretion of sodium will be increased. In contrast, after acute expansion of the extracellular space with saline both the absolute and fractional excretion of sodium by the previously-obstructed kidney is greater than in the contralateral kidney. In this setting there is a greater increase in the sodium excretion from the previously-obstructed kidney, than from the contralateral kidney [Wilson DR 1974].

Bilateral ureteral obstruction. The release of bilateral ureteral obstruction results in a dramatic increase in sodium and water excretion in the urine in experimental animals [Jaenike J 1972, McDougal WS 1972, Yarger WE 1972]. Absolute sodium and water excretion may increase up to fivefold, and because there is a concomitant reduction in glomerular filtration rate fractional excretion is increased.
even more. The amount of water excreted may be elevated by ninefold. This does not appear to be a result purely of volume expansion following bilateral ureteral obstruction since in most experimental studies the animals are fasted following obstruction and lose weight. Instead there appears to be a decrease in the reabsorption of sodium and water by one or more segments of the renal tubule.

The mechanism responsible for this alteration in sodium reabsorption is not entirely clear. Increases in ureteral pressure are not responsible since this tends to increase the reabsorption of sodium and water [Fulop M 1970]. Although the post-obstructive diuresis is less following unilateral ureteral obstruction, the number of kidneys obstructed does not condition this phenomena since a post-obstructive diuresis occurs after release of ureteral obstruction if the opposite kidney has been severely damaged or removed surgically. Thus it is the period of anuria which appears to condition the diuretic response of the kidney, and by implication it has been suggested that natriuretic factors accumulate during the period of anuria. This is discussed more fully subsequently. In contrast to unilateral ureteral obstruction, following the release of bilateral ureteral obstruction there is a decrease in sodium reabsorption in both the proximal and distal tubule [Yarger WE 1972]. Whether salt and water reabsorption in the collecting duct is impaired following ureteral obstruction is unclear with micropuncture and microcatheterization studies yielding conflicting results [Buerkert J 1977, Sonnenberg H].

1.4.2 Concentrating ability following ureteral obstruction.

Following ureteral obstruction the ability to concentrate the urine is markedly impaired with maximum values of 350-400 mosmol reported in the rat [Buerkert J 1978]. As discussed above salt reabsorption by the thick ascending limb is decreased,
resulting in a loss of medullary tonicity which diminishes water abstraction from the descending limb of Henle's loop. The ability to dilute the urine is less markedly affected, however. Since dilution of the urine occurs mainly in the thick ascending limb of Henle's loop secondary to a greater reabsorption of solute than water in this segment, the differences between concentrating and diluting ability suggest that factors other than decreased solute reabsorption by the thick ascending limb play a role in the concentrating defect. One such factor may include the increase in medullary blood flow in obstruction which combined with an overall decrease in the glomerular filtration rate in deep nephrons would lead to solute depletion in the medullary interstitium. In addition, there is decreased response of the cortical collecting duct to the action of antidiuretic hormone (see section 1.4.4).

The differences in salt and water excretion following the release of unilateral or bilateral ureteral obstruction could be due to differences in intrinsic changes within the nephron in response to ureteral obstruction or to a difference in extrinsic factors between the two models, or a combination of both.

In rabbits, no intrinsic differences in the function of isolated nephron segments between nephrons isolated from animals with unilateral or bilateral ureteral obstruction could be demonstrated, although in both cases the nephrons behaved differently from nephrons isolated from normal kidneys [Hanley MJ 1982]. Reabsorption of salt and water by proximal tubules is unchanged, whereas that by the thick ascending limb of the loop of Henle and by juxtamedullary proximal tubules are decreased following both unilateral and bilateral obstruction [Hanley MJ 1982]. This would suggest that the observed differences in salt and water reabsorption in surface nephrons between bilateral and unilateral ureteral obstruction-unchanged in the former but increased in
the latter is due to extrinsic factors, rather than to differences in intrinsic changes within the nephron.

Part of the differences observed after release of unilateral or bilateral ureteral obstruction may be due to dissimilar haemodynamic responses in the two settings. Renal blood flow is reduced in both models of obstruction, but returns towards normal following the release of bilateral obstruction, whereas following the release of unilateral obstruction renal blood flow remains depressed and its distribution is shifted towards the inner cortex [Seigel NJ 1977]. Thus, the filtration fraction of outer cortical nephrons will be high following the release of unilateral (but not bilateral) ureteral obstruction, and the attendant increase in peritubular oncotic pressure could contribute to the increased fractional reabsorption of fluid observed in superficial proximal and distal tubules following unilateral ureteral obstruction.

In bilateral ureteral obstruction accumulation of natriuretic factors during the period of anuria may contribute to the increased excretion of salt and water observed. Urea acting as an osmotic agent has been implicated, since urea infusion to animals following release of unilateral ureteral obstruction can cause a diuresis with fractional excretion rates of sodium reaching 9% [Harris RH 1975]. Care must be taken to replace all solute and water losses from the contralateral kidney for this effect to be seen. The effect is much greater when urine is rein infused instead of simply urea, suggesting that there may be additional natriuretic factors in urine other than urea [Harris RH 1975, Harris RH 1977].

Protein intake has been found to condition the diuresis that is seen after bilateral ureteral obstruction [Purkerson ML 1984], with animals fed a high protein diet demonstrating a greater diuresis. In these experiments increased excretion of urea
accounted for only part of the enhanced diuresis and natriuresis in the high protein fed animals, again suggesting a role for the accumulation of other natriuretic factors during a period of obstruction.

There also appears to be a circulating factor that accumulates in response to complete bilateral ureteral obstruction, since a diuresis and natriuresis comparable to that seen after release of bilateral ureteral obstruction can be elicited in normal rats by cross circulation with rats with complete ureteral obstruction of 24 hours duration [Wilson DR 1976]. This circulating factor may well be atrial peptide, since serum levels of this peptide are elevated in rats with bilateral ureteral obstruction compared to control rats or rats with unilateral ureteral obstruction [Purkerson ML 1989c]. Furthermore, infusion of heparin, which has been shown to block the biological effects of atrial peptide reduces the natriuresis and diuresis that is seen following release of bilateral ureteral obstruction. Calculations have shown that the elevated levels of atrial peptide following bilateral ureteral obstruction could account for up to one-fourth of the diuresis and natriuresis that occurs. The mechanisms responsible for the increased levels of atrial peptide following bilateral ureteral obstruction remain to be determined, but may be due to expansion of extracellular fluid volume, decreased metabolism of atrial peptide by the kidney, and perhaps to other mechanisms such as changes in atrial pressure and/or the circulating plasma levels of antidiuretic hormone and/or angiotensin II.

1.4.3 Alterations in renal potassium handling.

The effect of obstruction on renal potassium handling depends on whether the obstruction is unilateral or bilateral. In the presence of a normal functioning contralateral kidney potassium excretion is reduced following relief of obstruction,
either in proportion to, or perhaps greater than the fall in glomerular filtration rate i.e. fractional excretion of potassium is unaltered or slightly reduced [Buerkert J 1979]. Renal potassium excretion does not increase normally in response to sodium sulphate administration [Thirakomen K 1976]. These data would suggest that there is a defect in the distal potassium secretory mechanism following unilateral obstruction, which may possibly be secondary to an unresponsiveness of that segment of the nephron to aldosterone. In contrast, following release of bilateral ureteral obstruction there is a marked increase in the net and fractional excretion of potassium [Buerkert J 1977, McDougal WS 1972]. The major mechanism by which potassium losses occur in this setting is an increased delivery of sodium to the distal tubule, resulting in an accelerated sodium potassium exchange. In support of this micropuncture studies have shown that delivery of potassium to end accessible sites of the proximal tubule and to the bend of the loop of Henle of deep nephrons is not greater than values obtained in control studies, while delivery to the base of the terminal segment of the collecting duct, beyond which no potassium secretion has been found, is markedly increased [Buerkert J 1977]. Micropatheterization studies have shown that fractional delivery of potassium to any site along the medullary collecting duct increases as a function of distance from the cortical junction, which suggests that the medullary collecting duct is the site of altered potassium handling [Sonnenberg H 1976]. The hyperkalaemia and the fall in bicarbonate concentrations that occur during the period of anuria in bilateral ureteral obstruction may stimulate renal acid and potassium excretion.
Altered hormone responsiveness and sensitivity of the obstructed kidney.

Altered renal response to the hormones aldosterone and antidiuretic hormone may play a role in the altered handling of water, sodium and potassium which is seen following ureteral obstruction.

**Aldosterone.** Following unilateral ureteral obstruction in dogs there appears to be an impairment of both potassium secretion and mineralocorticoid responsive sodium reabsorption [Thirakomen K 1976]. A hyperkalaemic distal renal tubular acidosis is common in patients with obstructive uropathy suggesting that there is an end organ insensitivity to aldosterone.

**Antidiuretic hormone (ADH or vasopressin).** An unresponsiveness of the collecting duct to ADH has been suggested to contribute to the concentrating defect that is seen following ureteral obstruction. The cyclic AMP response to exogenous ADH is markedly blunted in rats with bilateral ureteral obstruction compared to non-obstructed rats [Beck N 1975, Schlondorff D 1983]. In addition, ADH induced osmotic water flow has been shown to be significantly impaired in cortical collecting tubules isolated from obstructed kidneys of rabbits [Campbell HT 1985, Hanley MJ 1982].

The mechanisms responsible for the alterations in hormone sensitivity are not immediately apparent. The decreased activation of the adenylate cyclase system may be due to alterations in the guanine nucleotide regulatory proteins, with $N_s$, the stimulatory component being decreased and $N_i$, the inhibitory component, being increased following ureteral obstruction. In addition, another second messenger pathway, the inositide pathway, may be impaired following ureteral obstruction. Thus, the generation of both phosphatidylinositol 1,4,5 triphosphate (IP$_3$) which mobilizes
calcium from intracellular organelles as well as diglyceride, which can activate protein kinase C and thus effect the phosphorylation of proteins, may be impaired. Indeed the phosphatidylinositol 4,5 bisphosphate (PIP$_2$), the precursor of IP$_3$, and diglyceride is decreased in the obstructed kidney of rats with unilateral ureteral obstruction compared to the contralateral kidney.

1.4.5 Recovery of tubular function following ureteral obstruction.

Whilst the effects of ureteral obstruction on changes in tubular function in the immediate period that follows relief of obstruction have been extensively studied, the long-term effects of ureteral obstruction on tubular function are less well known. Recent studies in which tubular function was studied following the release of 24 hours of unilateral ureteral obstruction in rats, have revealed that abnormalities of tubular function persist beyond a time (14 days) when whole kidney glomerular filtration rate has returned to normal [Bander SJ 1985]. Urine osmolality was consistently lower in the post-obstructed kidney up to 60 days following release of the obstruction. Net acid secretion is decreased and ammonium excretion is less in the post-obstructed kidney, the latter being due to a defect in ammonia re-entrainment in the collecting duct. Urine pH remained higher in the post-obstructed kidney up to 14 days following the release of the obstruction and potassium excretion remained lower. These observations on tubular function following release of ureteral obstruction are consistent with persistent alterations in distal tubular/collecting duct function and/or a loss in functioning juxtaglomerular nephrons following the release of the obstruction.
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1.5 Histological changes in the kidney following urinary tract obstruction.

The morphological alterations in renal architecture caused by obstruction can be predicted from the major causes of parenchymal damage: 1) increased ureteral pressure, 2) decreased renal blood flow, 3) obstruction to venous drainage, 4) invasion by macrophages and lymphocytes, and 5) bacterial infection. Grossly hydronephrotic kidneys have a widely dilated renal pelvis with the renal papillae either flattened or hollowed out. The ducts of Bellini are the first renal structures to be affected. Subsequently, other papillary structures are involved. Ultimately, there is an encroachment upon renal cortical tissue, which in advanced cases may be reduced to a thin rim of renal tissue surrounding a large sacular ureteral pelvis.

Histologically, as seen in experimental animals the early changes of hydronephrosis consist of dilatation of the tubular system, predominantly the collecting duct and distal tubular segments. Subsequently, cellular flattening and atrophy of the cells lining the proximal tubule occur [Deming CL 1951, Himan F 1945, Sheehan JH 1959, Shimamura T 1966, Strong KC 1940]. In most instances there is a tendency for glomerular preservation. Thus, Bowman's space may be dilated and ultimately some periglomerular fibrosis may develop. Similar morphological changes have been described after single nephron obstruction [Tanner GA 1989]. Vascular stretching and other arterial changes consisting of multiple ruptures of the internal elastica lamina have also been described in arteries of the pelvis [Altschul R 1953]. Sequelae of tubular ischemia, the consequence of the combined effects of a decreased renal blood flow and obstruction, are the development of interstitial fibrosis and mononuclear infiltration [Davis BB 1983]. The mechanisms underlying the development of this infiltrate have not been elucidated but may relate
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to the liberation of a macrophage-specific chemoattractant(s) released by the kidney during obstruction. In addition, infection (pyelonephritis) may play a role in the development of parenchymal fibrosis.

1.6 **Role of infiltrating cells in post-obstructive renal haemodynamic changes.**

Prior to the publication of the work presented in this thesis little was known about the role of infiltrating cells in the pathogenesis of obstructive nephropathy. Chronic renal obstruction in rabbits had been reported to be associated with a proliferation of interstitial fibroblasts and an infiltration of mononuclear cells [Nagle RB 1973, Nagle RB 1976]. This interstitial cell infiltrate had been linked to the increase in prostaglandin E\(_2\) production by the chronically hydronephrotic rabbit kidney [Davis BB 1983]. Furthermore, it had been postulated that the infiltration of the renal parenchyma by mononuclear cells was necessary for the enhanced release of thromboxane A\(_2\) and prostaglandin E\(_2\) that is seen following ureteral obstruction in response to bradykinin [Okegawa T 1983] or endotoxin [Lefkowith JB 1984]. This was based on the observation that the administration of nitrogen mustard to the rabbits so as to render them leukopaenic significantly blunts the enhanced prostanoid release in response to bradykinin or endotoxin following ureteral obstruction.
CHAPTER 2

EXPERIMENTAL MODELS OF OBSTRUCTIVE NEPHROPATHY

2.1 Models of obstructive nephropathy.

Studies on ureteral obstruction have attempted to define two points, namely i) the glomerular haemodynamic and tubular abnormalities that occur as a result of an obstruction to the drainage system of the kidney. These changes appear to be functional changes with little or no alteration in renal structure, and ii) the long term consequences of ureteral obstruction on kidney in which there are marked alterations in renal structure as well as function. Until recently there has been little data to suggest how the acute alterations in nephron function that result from obstruction eventually lead to an irreversible loss of renal mass.

Various experimental manipulations and conditions have been employed in an attempt to define the pathophysiological consequences of ureteral obstruction. The following must be considered when comparing different studies on this subject.

2.1.1 Species of Animal.

The majority of studies have been performed on rats; in this species it is possible to perform micropuncture studies which have allowed the single nephron glomerular haemodynamic changes in obstruction to be determined [Dal Canton 1979]. A variety of other species have been studied and although there is little species to species variation in the renal response to obstruction, minor differences have been reported. For example thromboxane production is less marked in the cat kidney in response to ureteral obstruction than in other species [Reginold DF 1981].
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Technical considerations, principally related to the size of the animal have also
determined the type of experiments it is possible to perform in a reproducible and
standardized fashion. For example sheep have been used to study the complex
pathophysiology of congenital obstructive uropathy. The stability of the pregnant
ovine uterus makes it suitable for intrauterine surgery, and thus study of the effect of
foetal ureteric obstruction on the development and function on the kidney [Ward RM
1989, Pringle KC 1988].

2.1.2 Age of Animal.

It is clear that the consequences of ureteral obstruction vary according to the
age at which the obstruction occurs. In the rat ureteral obstruction during early
development results in an activation of renin gene expression [El Dahr SS 1990a],
where as this does not occur in the mature animal [El Dahr SS 1990b]. Ureteral
obstruction during the period of glomerulogenesis appears to have a permanent
adverse effect on the renal development in most species studied. There is loss of
cortical differentiation in rats [Josephson S 1989], a loss in glomerular number in
rabbits [McVary KT 1989], and renal dysplasia in sheep [Pringle KC 1988].
Obstruction of the fully developed kidney does not share this potential for causing
such severe disruption, and recovery of whole kidney function following release of
relatively short periods (<10 days) of partial obstruction appears to be complete in the
adult animal in contrast to the neonate [Chevalier RL 1988]. In this context it must
be noted that the duration and timing of glomerulogenesis varies significantly between
species: in rodents it is continued into the neonatal period, where as in many larger
animals (including man) it is complete during intrauterine life. Thus care is needed
in extrapolating the effects of renal obstruction induced in young rodents to the effects
of non-congenital renal obstruction in man. Conversely neonatal rodents (with the attendant ease of operating ex-utero), can be used as a model of congenital obstructive uropathy in man. In this regard the North American opossum, Didelphis virginiana, has been used as a model for studying the effects of early fetal urinary obstruction on subsequent renal development. Ureteral obstruction results in renal dysplasia in this marsupial which has a typically mammalian kidney but in whom renal genesis occurs ex utero [Steinhardt GF 1988a].

2.1.3 Degree and Duration of Obstruction.

The majority of studies have examined the effect of acute and complete ureteral obstruction. This can simply be achieved in small experimental animals by securing a silk ligature around the ureter and gives a very constant lesion. Partial ureteric obstruction in smaller animals is more difficult to standardize, but has been produced by incomplete external compression of the ureter, either with a ligature or plastic ring, or by embedding the ureter in the psoas muscle [Stenberg A 1988]. In larger animals such as dogs it is possible to create partial ureteric obstruction by insertion of an obstructing stent of a fixed diameter into the ureter [Leahy AL 1989]. Furthermore, altering the internal diameter of the stents allows variation in the degree of obstruction [Ryan PC 1987], which can then be assessed using an indwelling ureteric catheter and a Whitaker test [Ryan PC 1989]. Angiographic balloon catheters can also be inserted into the ureter to study the effects of ureteral obstruction (balloon inflation) and release of obstruction (balloon deflation) [Crowley AR 1990].

The duration of the obstruction and the length of time allowed to elapse following release of the obstruction both condition the result of renal function tests. In the developing foetal lamb kidney recovery of renal function is inversely
CHAPTER 2 EXPERIMENTAL MODELS

proportional to duration of obstruction and directly proportional to the duration of incept decompression. Early in uterine decompression prevents or greatly ameliorates the development of renal dysplasia although, some post-obstructive changes persist if the in utero obstruction has been prolonged [Glick PL 1984]. In adult rats with complete ureteral obstruction, there appears to be two distinct phases; i) a "destructive phase" two to six weeks after obstruction when there is loss of renal tissue and ii) a "steady-state phase", when renal mass is constant. Relief of obstruction in this late or steady-state phase will not result in structural recovery of the kidney [Gonnermann D 1990].

It should also be noted that different aspects of nephron function will recover at a different rate following a given period of ureteral obstruction. For example, following 24 hours of complete ureteral obstruction in the rat, tubular function remains abnormal long after whole kidney glomerular filtration rate has recovered [Bander SJ 1985].

2.1.4 Unilateral or Bilateral Obstruction.

The renal response to ureteral obstruction is modified by whether one or both ureters are obstructed. This difference in response relates to the presence or not of a functioning contralateral "control" kidney. In addition, when both kidneys are obstructed the animal rapidly becomes uremic and thus only short term studies i.e. 24 hours of obstruction, can be performed.

Unilateral ureteral occlusion has less profound effects on tubular pressure than bilateral obstruction; as a result, a greater fall in glomerular filtration would be expected following bilateral ureteral obstruction than unilateral ureteral obstruction. However, inulin and p-aminohippurate clearance are significantly higher following the
CHAPTER 2 EXPERIMENTAL MODELS

release of 24 hours of bilateral as opposed to unilateral ureteral obstruction. This may reflect the presence of a greater intraglomerular capillary pressure in animals with bilateral obstruction than in those with unilateral obstruction as the latter model is associated with intense afferent arteriolar constriction [Dal Canton 1979]. In addition, after 24 hours of obstruction the number of filtering nephrons is greater prior to and after release of obstruction in rats with bilateral ureteral obstruction than in those with unilateral ureteral obstruction [Buerkert J 1983]. The mechanisms responsible for these differences in renal haemodynamics are not well understood. Plasma levels of atrial peptide are higher in animals with bilateral ureteral obstruction than in those with unilateral ureteral obstruction [Himmelstein SI 1990, Purkerson ML 1989b]. Atrial peptide causes pre-glomerular vasodilatation and post-glomerular vasoconstriction [Marin-Grez M 1986], and has been demonstrated to increase Kf in the isolated perfused glomerulus preparation [Fried TA 1986], and thus could account for the observed differences between the two models.

2.1.5 Experimental Conditions.

The response to ureteral obstruction can be modified by alterations in the experimental conditions employed and this may account for discrepant results reported by different laboratories. For example, preconditioning rats with a low protein diet decreases the renal synthesis of thromboxane A2 and ameliorates the decline in glomerular filtration rate and renal plasma flow following ureteral obstruction [Ichikawa I 1985]. Variations in fluid replacement regimes may also have an effect with volume loading significantly increasing post-obstructive glomerular filtration rate [Wilson DR 1974].
CHAPTER 3

METHODS

3.1 Animal preparation.

The experimental model of ureteral obstruction used in this series of experiments was that of acute (i.e. less than 24 hours) ureteral obstruction in the adult rat. Both bilateral and unilateral ureteral obstruction were studied.

The experimental protocols were conducted within the requirements of the Federal Government of USA regarding the humane care of laboratory animals and in adherence with the Declaration of Helsinki and to the principles of the American Physiological Society regarding the care of laboratory animals. In addition, all experimental protocols were performed with the prior approval of and in accordance with the guidelines laid down by the "Committee for the Humane Care of Laboratory Animals", Washington University, St Louis, MO, USA, which was the host institution.

3.1.1 Animals.

Lewis male rats were used in all studies. They were obtained from Harlan Sprague-Dawley (Indianapolis, Indiana, USA). Rats were at least 3 months old and weighed at least 200 grams prior to study.

3.1.2 Diet.

Rats were fed a normal rat chow in dry pellet form, from weaning (Ralston Purina, St Louis MO, USA). The diet contained 22% protein and 4.5% fat. The animals were allowed free access to food and tap water prior to the study period. Food consumption was not routinely monitored prior to the experiments but was typically of the order of 15-20 g/day.
CHAPTER 3

METHODOLOGY

3.1.3 **Animal Housing.**

Prior to study the animals were housed in groups of no more than 6, with temperature and humidity being maintained at 18-22°C and less than 50% respectively. They were exposed to a 12 hour light/dark cycle.

3.2 **Operative procedures.**

3.2.2 **Induction of ureteral obstruction.**

The animals were anaesthetized with Ether (Sigma, St Louis MO, USA), in an ether box and anaesthesia maintained with a cone of ether placed over the animals nose. All operative procedures were performed in a fume hood with face air movement of 100 cfm to avoid operator exposure to ether. The abdomen was shaved and the skin swabbed with ethyl alcohol. A small (2cm) lower mid-line incision, was then made in the skin and the recti muscles separated in the mid-line to expose the peritoneal cavity. The small bowel was then gently manipulated cranially using a sterile gauze swab soaked in 0.9% saline, thus exposing the retroperitoneal area. The left and right ureters could then be easily identified. The ureters appear as approximately 1mm diameter structures with longitudinal blood vessels, running either side of the rectum, and characteristically are seen to undergo peristalsis.

3.2.2.1 **Sham Operation.**

The ureters were gently manipulated with a swab, but no ligature was applied.

3.2.2.2 **Unilateral Ureteral Obstruction (ULO).**

A sterile 4/0 silk ligature was passed around either the left or right ureter at the junction of the upper 2/3 and lower 1/3, and tied. The contralateral ureter was gently manipulated with a swab, but no ligature was applied.
3.2.2.3 **Bilateral Ureteral Obstruction (BUO)**

A sterile 4/0 silk ligature was passed around each ureter in turn at the junction of the upper 2/3 and lower 1/3, and tied.

3.2.2.4 **Reversible Ureteral Obstruction.**

The ureter was gently mobilized from the retroperitoneal structures. A piece of bisected polyethylene tubing (PE50), 5 mm in length, was passed around the ureter, and secured with a 4-0 silk ligature tied tightly so as to occlude the ureter. This method prevented the ureter from being irreversibly crushed by the obstructing ligature and allowed easy subsequent release of the obstruction by cutting the ligature and removing the polyethylene tubing.

The abdominal swab was then removed and the small bowel gently repositioned. The subcutaneous tissue was then closed with a continuous suture of 3-0 black silk, and the skin was closed by the application of stainless steel clips. The operative procedure typically took less than 5 minutes from the induction of anaesthesia.

The rats were returned to cages and were typically fully conscious in 30 minutes. In the case of BUO, as renal function had effectively been abolished they were given neither access to tap water nor food, in order to prevent volume overload. Following UUO, as the animals still had a single functioning kidney they were allowed free access to tap water but no food.
CHAPTER 3 METHODS

3.3 Renal clearance measurements.

3.3.1 Preparation.

The animals were anaesthetized in an ether box and anaesthesia maintained with a cone of ether placed over the animals nose. The abdomen was reopened through the previous incision which was extended cranially by 1.5 cm. The small bowel contents were moved so as to reveal the ureters.

In the animals which had undergone BUO, a single obstructed ureter was cannulated above the tie on one side so as to effect unilateral release of the obstruction. The ureteric catheter was constructed from a 1 cm piece of PE-50 tubing tied into a silastic tubing. A small incision was made in the obstructed ureter (which was significantly dilated) and the PE-50 tubing was inserted and secured with two 4-0 silk ligatures. The flexible silastic tubing was then led to the exterior of the animal to allow collection of urine from the previously obstructed kidney. In the case of the sham operated controls, a single unobstructed ureter was cannulated. As this ureter was not dilated, a piece of PE-10 tubing was used to cannulate the ureter. Great care was taken not to traumatize the ureter and thus induce haematuria. In the case of the animals which had undergone UUO, both the previously obstructed and the unobstructed ureters were cannulated to allow collection of urine from the post-obstructed and control kidneys.

The abdomen was then closed en-mass with a single running suture of 4-0 silk, taking care not to kink the silastic catheters draining from the ureters.

A tail vein catheter was inserted to allow infusion of solutions. This was constructed from PE-50 tubing into which a 23 gauge needle had been secured with dental wax. The needle was inserted into one of the lateral tail veins of the rat by
direct puncture and secured and splinted in place.

A femoral artery catheter was placed in order to allow blood samples to be taken. Either the left or right femoral artery was exposed and dissected from the vein and nerve. A catheter constructed of PE-10 tubing was inserted into the femoral artery to allow collection of blood samples and the groin incision was closed. At the end of the femoral artery catheter a piece of flexible (wire insulation) tubing was attached to allow the tubing to be clamped without causing permanent damage.

The rats were secured in individual plastic holders (Plexiglas), which were specially designed to allow the animals to recover whilst being restrained. In this way clearance studies could be performed in the awake state.

3.3.2 Clearance Measurements.

The animals were allowed to recover for three hours in the plastic restrainers before they were studied in the awake state.

One hour prior to study (three hours post release) a priming dose of chemical inulin (Fisher Co, St. Louis, MO, USA) (40 mg/ml) and chemical p-aminohippurate (PAH) (Merck, Sharp and Dohme, West Point, PA, USA) (2 mg/ml) was given in 0.9 ml of 0.9% saline over one minute and constant infusions started to maintain plasma levels of inulin at 70-120 mg/100 ml and PAH levels at 0.8-2.0 mg/100 ml. The infusate was administered at a rate of 39 µl/min. In sham operated animals or animals with UUO, (i.e. those with one or more functioning kidneys) the infusate concentrations required to maintain these plasma concentrations of inulin and PAH were 40 mg/ml of inulin and 2 mg/ml of PAH. In animals with unilateral release of BUO the infusate concentrations required to maintain these plasma concentrations of inulin and PAH were 20 mg/ml 1 mg/ml respectively.
A one hour equilibration period was allowed before clearance were performed. Thus, a four hour period following release of the obstruction elapsed before the clearance studies were performed. Two timed (20-30 minute) urine collections were made into pre weighed tubes stored on ice. The volume of urine collected was determined gravimetrically. Blood was collected via the femoral artery catheter at the beginning, midpoint and end of each collection period into pre-heparinized microhaematocrit tubes. A total of 300 µl of blood (5 haematocrit tubes) was collected per period and replaced with an equivalent volume of 0.9% saline. The haematocrit of the arterial blood samples was determined after centrifugation for 5 minutes. An average value of haematocrit for each clearance period was determined from the 5 tubes of blood. The plasma was then separated from the blood cells and stored overnight at 4°C for analysis the following day (see section 3.4).

An aliquot of each urine sample was stored at -70°C for subsequent analysis of thromboxane B2 (the stable metabolite of thromboxane A2) and prostaglandin E2. If the urine was bloody, it was not used for prostanoid analysis because of contamination caused by prostanoids particularly thromboxane B2 derived from circulating cells such as platelets.

The remaining urine was stored overnight at 4°C and analyzed the following day as described in section 3.4.
3.4 Analytical techniques.

Inulin, PAH, sodium and potassium, urea and creatinine were routinely measured in duplicate on the samples obtained from the rats during the clearance experiments.

3.4.1 Inulin.

Inulin in plasma and urine was determined using the method of Fuhr [Fuhr J 1955]. (see Appendix 1).

3.4.2 PAH.

PAH was measured by a modification of the method of Smith et al [Smith HW 1945]. (see Appendix 2).

3.4.3 Urea and Electrolytes and Creatinine.

Sodium and potassium were measured in plasma and urine by standard flame photometry (Instrumentation Laboratory Inc., Lexington, MA, USA). Blood urea nitrogen and plasma creatinine, were measured using standard colorimetric methods on a Multistat III plus autoanalyser (Instrumentation Laboratory Lexington MA, USA).

3.4.4 Urinary Prostanoids.

Urinary thromboxane B₂ (the stable metabolite of thromboxane A₂) and prostaglandin E₂ were assayed in duplicate respectively, by radio-immunoassay using antisera raised in rabbits using methodology modified from Reingold et al [Reginold DF 1981]. Cross reactivities with other prostaglandins at 50% displacement were less than 0.4% for the prostaglandin E₂ antiserum and less than 0.025% for the thromboxane B₂ antiserum. (see Appendix 3).
3.5 Clearance Calculations

Calculations of glomerular filtration rate and renal plasma flow were made according to standard formulae [Schuster VL 1985]. The following were routinely calculated.

1. Glomerular Filtration Rate (GFR) = \( C_u = \frac{(U_u \times v)}{P_u} \) (ml/min)
2. Renal Plasma Flow (RPF) = \( C_{PAH} = \frac{(U_{PAH} \times v)}{P_{PAH}} \) (ml/min)

More accurately RPF = \( v(U_{PAH} - R_{PAH})/(A_{PAH} - R_{PAH}) \) where A and R represent the arterial and venous concentrations respectively (the Fick principle). \( C_{PAH} \) may be used as an estimate of renal plasma flow as the extraction of PAH by the kidney approximates to 100% (Extraction ratio \( E_{PAH} = (Art_{PAH} - Venous_{PAH})/Art_{PAH} = 1 \)). Thus renal vein PAH is negligible compared to renal artery PAH and the equation for RPF reduces to \( C_{PAH} \). Measured values for \( E_{PAH} \) would suggest the value is in fact nearer 0.9 [Smith HW 1951], i.e. \( \approx 90\% \) of renal artery PAH is extracted in a single pass through the kidney, but the error introduced by this is small and renal vein PAH is not routinely measured. As the tubular capacity for secretion of PAH is limited, \( E_{PAH} \) will fall if the plasma concentrations of PAH become too high. In order to avoid this, in the current experiments the protocol was designed to keep values for \( P_{PAH} \) below 2.0mg% and any experiments in which values exceeded this were not used for analysis.

3. Renal Blood Flow = \( \text{RPF}/(1 - \text{Hct}) \)
4. Filtration Fraction = \( \text{GFR}/\text{RPF} = C_u/C_{PAH} \)
5. Urinary sodium excretion \( v \times U_{Na} \) (\( \mu \text{mol/min} \))
6. Urinary potassium excretion \( v \times U_{K} \) (\( \mu \text{mol/min} \))
CHAPTER 3 METHODS

7. Fractional sodium excretion \( FE_{Na} = \frac{(U_{Na} \times P_{Na})}{(P_{Na} \times U_{Na})} \)

8. Fractional potassium excretion \( FE_{K} = \frac{(U_{K} \times P_{K})}{(P_{K} \times U_{K})} \)

9. Fractional water reabsorption \( FR_{WR} = \frac{(GFR - v)}{GFR} = 1 - \frac{(P_{U} / U_{Na})}{P_{Na}} \)

where:

\( v \) = urinary volume (\( \mu l/min \))

\( U_{Na} \) = Urinary inulin concentration  \( P_{Na} \) = Plasma inulin concentration

\( U_{PAH} \) = Urinary PAH concentration  \( P_{PAH} \) = Plasma PAH concentration

\( U_{Na} \) = Urinary sodium concentration  \( P_{Na} \) = Plasma sodium concentration

\( U_{K} \) = Urinary potassium concentration  \( P_{K} \) = Plasma potassium concentration

3.6 Labelling of Renal Tissue for Immune Cells

The animals were deeply anaesthetized with pentobarbitone (50mg/Kg Body Weight), the abdomen was (re)opened and the kidneys perfused in situ. The aorta was cannulated at its bifurcation with a 18 gauge needle and was then clamped above the liver. A small cut was then made in the inferior vena cava and the kidneys were perfused retrogradely up the aorta with phosphate buffered saline (PBS), at 37° C, to which was added papaverine at a concentration of 100 \( \mu g/ml \).

This technique was effective in thoroughly perfusing the obstructed kidney and thus eliminating the contribution of circulating leukocytes to the enumeration of the cells in the glomerulus and the interstitium. The animal was subsequently killed by exsanguination while under deep anaesthesia. The kidneys were then removed and processed in order to enumerate the leukocyte content according to one of the following techniques.
3.6.1 Quantification of Immune Cells in the Kidney.

a: Interstitium: The kidneys were bisected and the cortex separated from the medulla. A portion of dissected cortex or dissected medulla was minced through sieves with pore size of first, 500 microns, and then second, 250 microns using a rubber bung. The sieve-minced tissue was then placed in tubes, centrifuged for 3 minutes at 1000 rpm, washed in PBS and recentrifuged. The sediment was then weighed. All cell counts were normalized to the weight of the tissue yielding the cells. The interstitial tissues were then resuspended in digestion medium (5ml of digestion media per kidney suspension) for 30 minutes at room temperature on a mechanical rotator. The composition of the digestion medium is shown in Table 3.1.

Table 3.1 Composition of Digestion Medium.

<table>
<thead>
<tr>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hank's Balanced Salt Solution (HBSS), containing</td>
</tr>
<tr>
<td>1% Hepes Buffer,</td>
</tr>
<tr>
<td>Type 2 collagenase (500 µg/ml),</td>
</tr>
<tr>
<td>DNA-ase (0.01 mg/ml)</td>
</tr>
<tr>
<td>Soybean trypsin inhibitor (1 mg/ml)</td>
</tr>
</tbody>
</table>

The digested material was washed twice in PBS containing 2% fetal calf serum and then placed in PBS containing 1 mM EDTA for 10 minutes. The material which consisted of insoluble basement membrane and cell fragments was then washed once in PBS, and allowed to settle for 1 minute. The supernatant consisted of epithelial cells, leukocytes, and other components of the renal interstitium as single cells or cells in small clumps. Intact glomeruli are also obtained in the supernatant, as glomeruli are not digested by this protocol; leukocytes contained in those glomeruli were excluded from the count of interstitial leukocytes, as they were recorded separately.
Although some fragments of partially digested tubules settle to the bottom of the tube in the sedimentation step described above, labelling of the sediment for leukocytes revealed that greater than 95\% of the leukocytes in the total interstitial cell population were released by the enzymatic treatment described above and appeared in the supernatants. The single cell suspension was subsequently stained for leukocyte antigens, employing a variety of antisera outlined below, followed by FITC-labelled secondary antibody. Leukocytes were counted initially as a percentage of the total interstitial cell population, but because of concerns that obstructed interstitial cells, particularly epithelium, might be more susceptible to injury induced by the digestion medium, the leukocyte content of these tissues were subsequently expressed per gram wet weight. In this thesis the interstitial leukocyte infiltrate is expressed as cell number per gram of tissue. Since post-obstructed kidneys weigh more due to an increase in extracellular fluid, the increase in leukocyte numbers reported in this way would tend to understate the true influx of leukocytes.

Total leukocyte number was determined, together with a quantification of leukocyte subsets by using primary antibodies of appropriate specificities.

The following antibodies were employed:-

a) Mouse monoclonal anti-rat leukocyte common antigen (clone OX1), whose specificity has been detailed [Fabre JW 1977].

b) Mouse monoclonal anti-rat T lymphocyte (clone OX19), (Accurate Chemicals, New York, USA).

c) Mouse monoclonal anti-rat T lymphocyte, suppressor subset (clone OX8), (Accurate Chemicals, New York, USA).

d) Mouse monoclonal anti-rat T helper lymphocyte (clone OX22).
e) Polyvalent rabbit anti-rat macrophage antiserum absorbed against other leukocytes (Accurate Chemicals, New York, USA).

b: Glomerular: Glomeruli were isolated using previously described methods [Schreiner GF 1981]. The cortex was dissected from the medulla and passed through sieves with pore sizes graded from 250 micron, 150 micron to 75 micron. Using this method glomeruli are retained on the 75 micron sieve. They were then washed from the sieve with PBS into a 20 ml universal container. This resulted in a preparation of 93-97% glomeruli. The glomeruli were then centrifuged for 3 minutes at 1000 rpm and the supernatant aspirated. They were then resuspended in the digestion medium whose composition is described in Table 3.1. The glomeruli were rotated in this mixture at room temperature for 30 minutes, washed twice and then placed in one of the primary antibody preparations described above to label cells bearing macrophage or lymphocyte antigens. Primary antibodies were diluted in PBS to a concentration of 50 μg/ml. 5000 glomeruli were suspended in 350 μl of antibody solution. After 20 minutes at 4°C, the glomeruli were washed once, placed in PBS for 10 minutes to permit unbound antibody to diffuse out of the glomerulus, washed again and placed in a solution of secondary antibody. Secondary antibodies were FITC conjugates, directed at the appropriate specificity, at a concentration of 100 μg/ml in PBS. No labelling was observed with control primary antibody lacking the designated specificities or with secondary antibody exposure only. After washing, the glomeruli were fixed in 2% paraformaldehyde in PBS. The labelled cell content of isolated glomeruli was evaluated by microscopic examination with a Zeiss universal microscope. Cells were quantified by focusing through the glomeruli and counting...
CHAPTER 3 METHODS

cells as they appeared in the plane of focus. In all experiments leukocytes in 50-100 glomeruli were counted and their results averaged to give a mean glomerular leukocyte number. This technique has previously been shown to render intact glomeruli permeable to antibody, allowing in situ cells to be labelled [Schreiner GF 1981]. Because exposure to the enzymes is limited, overall glomerular architecture is not disturbed, permitting accurate evaluation of the leukocyte content of glomeruli on a per glomerulus basis.

c: Immunoperoxidase labelling of renal tissue: Frozen sections (6 μm) of kidneys harvested from control animals and animals with 24 hours of bilateral ureteral obstruction were fixed in chilled (4° C) acetone for 10 minutes and air-dried. Endogenous peroxidase activity was inhibited by immersion for 10 minutes in a solution consisting of 40 parts methanol, 9 parts H₂O, and 1 part H₂O₂ (30%). Endogenous biotin activity was inhibited by sequential 30 minute exposures to Avidin D and Biotin blocking solutions (Vector Laboratories, Burlingame, CA, USA). The sections were then placed in rabbit serum blocking solution (Zymed Laboratories, San Francisco, CA, USA), followed by anti-LC antibody (diluted 1:10 in PBS containing 3.5% bovine serum albumin) for 30 minutes. After washing, biotinylated rabbit anti-rat IgG (Vector Laboratories) affinity purified and adsorbed against mouse IgG, was added for 15 minutes. After washing, the slides were sequentially exposed to streptavidin-peroxidase followed by aminoethyl carbazole and hydrogen peroxide, as supplied by Zymed Laboratories. The sections were counterstained with haematoxylin and mounted under coverslips.

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3.6 Prostanoid production by isolated glomeruli.

Animals were anaesthetized with pentobarbitone (5 mg/100 g body weight i.p.) and the kidneys were perfused in situ with warm PBS. The cortex of the kidney was then separated on ice and the glomeruli were prepared using the sieving protocol previously described [Schreiner GF 1981]. Glomeruli which were retained by the 75μm sieve were suspended in HBSS and then washed twice with HBSS. The isolated glomeruli were then partially digested in HBSS containing 60 U/ml collagenase type 2 and 0.03 mg/ml DNase for 30 minutes at 37°C to remove Bowman's capsule. After treatment the preparations were washed three times with cold HBSS and then suspended in warm HBSS. Preparations contained more than 90% isolated glomeruli and most were free of Bowman's capsule. No differences in the purity of glomerular preparations were detected between sham-operated control rats and rats with bilateral ureteral obstruction.

Isolated glomeruli were pre-incubated in HBSS at 37°C for 30 minutes; 400 μl of the glomerular suspension were transferred into plastic centrifuge tubes and then incubated under continuous agitation (80 cycles/min) at 37°C for 60 minutes. The incubation was terminated by centrifugation (10,000 g for 1 min) at room temperature and the supernatants were stored at -70°C for subsequent determination of thromboxane B₂ and prostaglandin E₂ as described in Appendix 3. The protein content of the pelleted glomeruli was subsequently determined using the method of Lowry et al [Lowry OH 1951] (see Appendix 4). The values for glomerular eicosanoid production were corrected for the protein content of the glomeruli and expressed per mg protein per 60 min incubation.
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3.7 Chemotactic substance production by kidneys.

The chemotactic activity of kidney extracts was measured in the following way. Rats were anaesthetized with pentobarbitone 5 mg/100 g body weight given i.p. and the kidneys were perfused in situ with 60 ml of warmed (37°C) phosphate buffered saline, removed and decapsulated. The cortices were dissected away, gently minced, and placed in oxygenated RPMI-1640 tissue culture media (Washington University Tissue Culture Support Center, St. Louis, MO, USA) containing 0.25% fatty acid free albumin (Sigma Chemical, St. Louis, MO, USA), pH 7.1-7.2. The volume of media was adjusted so that there was 100 mg of cortex (wet weight) per ml of RPMI. This was incubated for one hour at 37°C under constant agitation. Centrifugation at 10,000 X G for 15 minutes was then performed to obtain a cell free supernatant, which was then assayed for chemotactic activity as described below.

3.7.1 Chemotaxis Assay.

The supernatants from the obstructed kidney and the contralateral control kidney were analyzed for chemotactic activity by the method of Falk, et. al. [Falk W 1980], using a multiwell microchemotaxis chamber (Neuroprobe, Cabin John, Md, USA). Supernatants containing the putative chemotactic factor were placed in the bottom wells of the chamber at variable dilutions. RPMI alone was placed in some wells to assess random cell migration. Each sample was tested in triplicate. The top wells of the chamber were filled with responder cells (see section 3.7.2) suspended in RPMI with 0.25% albumin.

A known positive chemotactic stimulus consisting of complement fragments (C5a/C5a des Arg) was also tested to ensure the integrity of the migrating cell population. Complement fragments were generated by exposing fresh rat serum to
zymosan (Sigma Chemical Co, St Louis MO, USA) for 30 minutes at 37°C. The
zymosan activated serum was used at a concentration of 5%.

The top and bottom wells were separated by a polyvinylpyrrolidone free
carbonate membrane containing 5 micron pores for macrophage migration or 3
micron pores for neutrophil (PMN) migration (Nucleopore, Pleasanton, Ca, USA).
These chambers were placed in a humidified incubator for four hours when testing for
macrophage migration (this was the optimal time as determined by preliminary
experiments) or for one hour when testing for PMN migration [Harvath L 1980]. At
the end of the incubation period, filters were removed, washed, fixed and stained
using a Diff-Quick Stain set (American Scientific Products, McGaw Park, Il, USA)
and allowed to dry. Cells which had migrated completely through the filter were
quantified using a Nikon Labophot microscope (x500) by counting five random fields.

Results are expressed as total cells migrating through the filter toward a
sample, or as net cells, defined as total cells which had migrated toward supernatants
from obstructed kidneys minus the number of cells migrating toward supernatants
from contralateral control kidneys. This definition of net cells controls for random
migration, and migration factors potentially released by the isolation and mechanical
disruption of renal tissue.

3.7.2 Preparation of responder cells

The responder cells were either:

1) Resident rat peritoneal macrophages. These were obtained by peritoneal lavage
of Lewis rats with RPMI. The cells were washed and resuspended in RPMI
with 0.25% albumin to a density of cells such that each well contained 35,000
macrophages. The purity of this preparation was 59% macrophages, with
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approximately 30% lymphocytes and the remainder being eosinophils and mast cells.

or:

2) Elicited rat polymorphonuclear leukocytes (PMN). These were obtained by lavage of the peritoneum of Lewis rats 12 hours after i.p. instillation of 0.5% sterile shellfish glycogen (Sigma Chemical, St. Louis, MO). The cells were washed and resuspended in RPMI with 0.25% albumin to a density of cells such that each well contained 50,000 PMNs. The purity was 79%.

3.8 Statistics.

All results in this thesis are expressed as mean ± standard error of the mean. Statistical analysis was performed using the unpaired Student's t-test. To compare values between multiple groups analysis of variance (ANOVA) was used. Correlations were calculated using linear regression analysis. Statistical analysis was performed using the C-Stat statistical software package (Ox-tech, UK version 1.0). A P value < 0.05 was considered statistically significant.
CHAPTER 4

THE FUNCTIONAL EFFECTS OF URETERAL OBSTRUCTION

4.1 Introduction.

It has been previously established that glomerular filtration rate declines immediately after the onset of obstruction [Wright F 1982], and that renal blood flow increases initially and then, after 2-3 hours, begins to diminish, declining to 40-70% of normal values by 24 hours [Wright F 1982, Klahr S 1983]. Tubular reabsorption of sodium and water is impaired on a sustained basis, even after the release of obstruction [Yarger WE 1982]. There is modulation of renal prostanoid production and the haemodynamic dysfunction has been attributed, in part, to augmented production of the vasoconstrictor prostanoid thromboxane A₂ [Morrison AR 1977, Yarger WE].

In this chapter the results of experiments designed to examine the haemodynamic and tubular effects of ureteral obstruction in the rat are reported. The time course of the renal haemodynamic changes in the model of bilateral ureteral obstruction are defined. In addition the relationship between the observed changes in renal function and the alteration in intrarenal prostanoid metabolism (particularly thromboxane A₂ and prostaglandin E₂) is examined.
CHAPTER 4 FUNCTIONAL EFFECTS

4.2 Methods.

4.2.1 The effects of varying durations of Bilateral Ureteral Obstruction.

Twenty-five male Lewis rats were used. Four groups of animals were studied following the induction of bilateral ureteral obstruction (as described in Chapter 3) for varying periods of time.

**Group I** Controls with 0 hours of BUO (no ureteral obstruction) (n=6). These animals underwent a sham laparotomy without ureteral obstruction 4 hours prior to preparation for clearance studies in order to reproduce the effect of the surgery alone on the renal clearance measurements and renal prostanoid production rates.

**Group II** 4 hours of BUO (n=6).

**Group III** 12 hours of BUO (n=7)

**Group IV** 24 hours of BUO (n=6)

Following the period of BUO, unilateral release of the obstruction was effected by insertion of PE50 tubing above the ligature on one side to drain the kidney; the rats were then prepared for clearance studies in the awake state as previously described in Chapter 3. The animals were studied 4 hours following the release of the ureteral obstruction. Inulin and PAH clearances as well as electrolyte excretion rates were determined. In addition prostanoid excretion rates were determined.

4.2.2 The effects of Unilateral Ureteral Obstruction.

In a further group of 8 rats the functional effects of 24 hours of unilateral ureteral obstruction were examined. The animals were prepared for study as described in Chapter 3. Inulin and PAH clearances as well as electrolyte excretion rates were measured from both the post obstructed kidney and contralateral control (non-obstructed) kidney.
CHAPTER 4  
FUNCTIONAL EFFECTS

4.3 Results.

4.3.1 The effects of Bilateral Ureteral Obstruction.

Inulin clearance was significantly diminished after 4 hours of obstruction and maximally depressed at 12 hours, after which no further decline occurred (Figure 4.1a).

Renal plasma flow as assessed by PAH clearance fell in parallel with the glomerular filtration rate following 4 hours of bilateral ureteral obstruction; thus filtration fraction was unaltered. However, with more prolonged periods of ureteral obstruction (12 and 24 hours), the fall in renal plasma flow was less marked than the fall in glomerular filtration rate (Figure 4.1b) and thus filtration fraction fell (Figure 4.1c).
Figure 4.1a  **Inulin clearance following ureteral obstruction.** Groups of rats were studied at timed intervals after the initiation of bilateral ureteral obstruction as described in the text. Controls values were obtained from one kidney of rats which had undergone a sham operation 4 hours previously (i.e. 0 hours of ureteral obstruction). Results are expressed as mean ± sem. (n=6 at all time points except 12 hours where n=7)
Figure 4.1b  **PAH clearance following ureteral obstruction.** Groups of rats were studied at timed intervals after the initiation of bilateral ureteral obstruction as described in the text. Controls values were obtained from one kidney of rats which had undergone a sham operation 4 hours previously (i.e. 0 hours of ureteral obstruction). Results are expressed as mean ± sem. (n=6 at all time points except 12 hours where n=7)
Figure 4.1c  **Filtration fraction following ureteral obstruction.** Groups of rats were studied at timed intervals after the initiation of bilateral ureteral obstruction as described in the text. Controls values were obtained from one kidney of rats which had undergone a sham operation 4 hours previously (i.e. 0 hours of ureteral obstruction). Results are expressed as mean ± sem. (n=6 at all time points except 12 hours where n=7)
The results for urine flow, fractional water excretion, and absolute and fractional excretion of sodium and potassium are summarized in Table 4.1. After unilateral release of only 4 hours of BUO the animals do not exhibit a post-obstructive diuresis or natriuresis when compared to sham operated controls. However, following greater than 12 hours of BUO there is a diuresis, natriuresis and kaliuresis, with significantly higher absolute and fractional excretions of water, sodium and potassium.

Table 4.1 The Effect of Varying Durations of BUO of Post-Obstructive Diuresis, Natriuresis and Kaliuresis.

<table>
<thead>
<tr>
<th>Duration of Bilateral Ureteral Obstruction</th>
<th>control</th>
<th>4 hours</th>
<th>12 hours</th>
<th>24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine Volume (μL/min)</td>
<td>20.78±</td>
<td>22.24±</td>
<td>35.08±</td>
<td>44.70±</td>
</tr>
<tr>
<td>UV_{Na} (μmol/min)</td>
<td>1.09</td>
<td>4.29</td>
<td>2.37**</td>
<td>4.64**</td>
</tr>
<tr>
<td>UV_{K} (μmol/min)</td>
<td>2.43±</td>
<td>1.21±</td>
<td>3.95±</td>
<td>3.97±</td>
</tr>
<tr>
<td>FE_{Na} (%)</td>
<td>1.03±</td>
<td>1.88±</td>
<td>1.61±</td>
<td>1.77±</td>
</tr>
<tr>
<td>FE_{K} (%)</td>
<td>0.11</td>
<td>0.22±</td>
<td>0.36*</td>
<td>0.46*</td>
</tr>
<tr>
<td>FE_{\text{ur}} (%)</td>
<td>1.44±</td>
<td>0.84±</td>
<td>5.73±</td>
<td>6.65±</td>
</tr>
<tr>
<td>FE_{\text{K}} (%)</td>
<td>0.18</td>
<td>0.29</td>
<td>0.44**</td>
<td>0.72**</td>
</tr>
<tr>
<td>FE_{\text{Buo}} (%)</td>
<td>22.49±</td>
<td>50.03±</td>
<td>81.72±</td>
<td>126.61±</td>
</tr>
<tr>
<td>FE_{\text{Buo}} (%)</td>
<td>1.48</td>
<td>6.35*</td>
<td>8.03**</td>
<td>9.87**</td>
</tr>
<tr>
<td>FE_{\text{HDO}} (%)</td>
<td>1.79±</td>
<td>2.28±</td>
<td>7.63±</td>
<td>10.84±</td>
</tr>
<tr>
<td>FE_{\text{HDO}} (%)</td>
<td>0.19</td>
<td>0.41</td>
<td>0.59**</td>
<td>1.03**</td>
</tr>
</tbody>
</table>

UV_{Na} = urinary sodium excretion, FE_{Na} = fractional sodium excretion
UV_{K} = urinary potassium excretion, FE_{K} = fractional potassium excretion
FE_{HDO} = fractional water excretion

* P < 0.01 vs control, ** P < 0.001 vs control.
Urinary thromboxane excretion, shown in Figure 4.2a and 4.2b, increased in a reciprocal pattern to the decline in glomerular filtration rate, with a maximum excretion at 12-24 hours. This pattern of a stepwise increment in thromboxane B₂ excretion was maintained whether the thromboxane excretion was expressed in absolute terms (Figure 4.2a) or normalized per ml of glomerular filtration rate (Figure 4.2b). A highly significant negative correlation was observed between the excretion rate of thromboxane B₂ and both the inulin and PAH clearances (Figure 4.3a and 4.3b).

Over the same time course the urinary excretion rate of the vasodilator prostanoid, prostaglandin E₂ also increased in a stepwise fashion (Figure 4.4). A significant correlation was observed between absolute prostaglandin E₂ excretion and urinary volume ($r = 0.788$, $P < 0.001$) and fractional water excretion ($r = 0.839$, $P < 0.001$) as well as absolute ($r = 0.652$, $P < 0.001$) and fractional sodium excretion ($r = 0.796$, $P < 0.001$). Figure 4.5a and b shows this relationship for fractional water and sodium excretion respectively. No correlation was observed between these parameters and thromboxane B₂ excretion rates.
Figure 4.2a  Urinary thromboxane excretion in rats at timed intervals after acute ureteral obstruction. Thromboxane B₂ excretion rate is shown in absolute amounts per group. The results expressed as mean ± sem. (n=6 at all time points except control where n=5).
Figure 4.2b  Urinary thromboxane excretion in rats at timed intervals after acute ureteral obstruction. Thromboxane B₂ excretion rate per group is normalized for the inulin clearance. The results expressed as mean ±sem. (n=6 at all time points except control where n=5).
Figure 4.3a Relationship between thromboxane B₂ excretion and inulin clearances. There is a strong and significant correlation between thromboxane B₂ excretion rates and inulin clearances at various times following ureteral obstruction. This suggests that the intrarenal generation of thromboxane A₂ may play a role in the altered haemodynamics seen following ureteral obstruction.
Figure 4.3b Relationship between thromboxane B₂ excretion and PAH clearances. There is a strong and significant correlation between thromboxane B₂ excretion rates and PAH clearances at various times following ureteral obstruction. This suggests that the intrarenal generation of thromboxane A₂ may play a role in the altered haemodynamics seen following ureteral obstruction.
Figure 4.4  Urinary prostaglandin E₂ excretion in rats at timed intervals after acute ureteral obstruction. The absolute prostaglandin E₂ excretion rate is given per group, with the results expressed as mean ± sem. (n=6 at all time points except control where n=5).
Figure 4.5a  The relationship between Prostaglandin E₂ excretion and fractional excretion of water. There is a strong and significant correlation between prostaglandin E₂ excretion rates and fractional water excretion at various times following ureteral obstruction. This suggests that the intrarenal generation of prostaglandin E₂ may play a role in the altered water handling which is seen following ureteral obstruction.
Figure 4.5b  The relationship between Prostaglandin $E_2$ excretion and fractional sodium excretion. There is a strong and significant correlation between prostaglandin $E_2$ excretion rates and fractional sodium excretion at various times following ureteral obstruction. This suggests that the intrarenal generation of prostaglandin $E_2$ may play a role in the altered sodium handling which is seen following ureteral obstruction.
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4.3.2 The effects of Unilateral Ureteral Obstruction.

24 hours of unilateral ureteral obstruction had a functionally similar effect to bilateral ureteral obstruction in that glomerular filtration rate was reduced to approximately one third of that of the non-obstructed contralateral kidney (2.19 ± 0.14 vs 5.34 ± 0.27 ml/min/kg BW P < 0.001). In addition PAH clearance fell (10.99 ± 0.65 vs 16.89 ±0.69 ml/min/kg BW P < 0.001). However, inulin clearance was decreased to a greater extent than that of PAH following unilateral ureteral obstruction resulting in a significant fall in filtration fraction (0.201 ± 0.012 vs 0.318 ± 0.011 P < 0.001).

The effect of 24 hours of unilateral ureteral obstruction on water and sodium excretion is shown in Table 4.2 where they are contrasted to the changes seen following bilateral ureteral obstruction. In contrast to the changes seen following 24 hours of bilateral ureteral obstruction there is no post-obstructive diuresis or natriuresis following the release of unilateral obstruction. This is despite comparable changes in the clearances of inulin and PAH in the two models.
### Table 4.2 Comparison between UUO and BUO on Post-Obstructive Renal Function, Diuresis, Natriuresis.

<table>
<thead>
<tr>
<th></th>
<th>UUO CCK</th>
<th>UUO POK</th>
<th>Control</th>
<th>BUO</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{na}}$ (ml/min/Kg BW)</td>
<td>5.34±0.27</td>
<td>2.19±0.14**</td>
<td>4.83±0.23</td>
<td>1.77±0.1**</td>
</tr>
<tr>
<td>$C_{\text{PAH}}$ (ml/min/Kg BW)</td>
<td>16.89±0.69</td>
<td>10.99±0.65**</td>
<td>17.07±0.42</td>
<td>8.23±0.4**</td>
</tr>
<tr>
<td>Urine Volume (µl/min)</td>
<td>21.0±3.7</td>
<td>8.0±1.3*</td>
<td>20.78±1.09</td>
<td>44.70±4.64**</td>
</tr>
<tr>
<td>$U_{\text{V,n}}$ (µmol/min)</td>
<td>2.7±0.56</td>
<td>1.02±0.21*</td>
<td>2.43±0.17</td>
<td>3.97±0.46*</td>
</tr>
<tr>
<td>$FE_{\text{Na}}$ (%)</td>
<td>1.19±0.25</td>
<td>1.16±0.33</td>
<td>1.44±0.18</td>
<td>6.65±0.72**</td>
</tr>
</tbody>
</table>

UV$_{\text{Na}}$ = urinary sodium excretion  
UUO = Unilateral ureteral obstruction  
CCK = Contralateral control kidney  
POK = Post-obstructed kidney  
BUO = Bilateral ureteral obstruction  

* $P < 0.02$ UUO POK vs UUO CCK  
** $P < 0.001$ UUO POK vs UUO CCK  
* $P < 0.01$ BUO vs control  
** $P < 0.001$ BUO vs control
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4.4 Discussion

Following ureteral obstruction, there is an initial prostaglandin-dependent increase in renal blood flow [Wright F 1982, Klahr S 1983, Allen JT 1978], which declines after 4 hours to reach 40-70% of normal values by 24 hours, due to progressive renal vasoconstriction. In the current study renal plasma flow as assessed by PAH clearance was first measured following 4 hours of ureteral obstruction. Thus the initial period of vasodilatation would have already passed and as such only the second phase of renal vasoconstriction was observed. The interpretation that there is renal vasoconstriction in this model depends on the assumption that a decrease in $C_{\text{PAH}}$ truly represents a decline in renal plasma flow. This in turn depends on the assumption that the extraction of PAH is unaltered by the process of ureteral obstruction. Since many tubular transport processes are altered following ureteral obstruction this assumption may not be valid. Indeed, there is evidence that the extraction of PAH is reduced following obstruction [Yarger WE 1974, Harris RH 1974, Jaenike J 1972]. This would result in $C_{\text{PAH}}$ falsely underestimating renal plasma flow and hence suggest the presence of more marked vasoconstriction than may exist.

The extraction of PAH was not directly measured in the studies presented in this thesis so the possibility exists that the degree of the fall in renal plasma flow following ureteral obstruction has been overestimated. However, it has been confirmed in other studies that ureteral obstruction causes a fall in renal plasma flow both by i) directly measuring the extraction of PAH in the rat [Harris RW 1974] and ii) the use of electromagnetic flow probes and radio-labelled microspheres to measure renal blood flow directly [Yarger WE 1974] in the dog.

These studies confirm that glomerular filtration declines following both
unilateral and bilateral ureteric obstruction. This has previously been shown to be due to a net decrease in the transglomerular pressure as a result of vasoconstriction of the renal arterioles and a decrease in $K_f$, the glomerular ultrafiltration coefficient (Ichikawa I 1985, Dal Canton 1980).

The data presented here is consistent with previous observations which demonstrated that $C_p$ is decreased to a greater extent than $C_{Pah}$ following ureteral obstruction (Yarger WE 1974, Harris RH 1974). This results in a decreased filtration fraction in the post-obstructed kidney and suggests that there is either diversion of blood to non-filtering areas of the kidney or that there is a reduction in the ultrafiltration coefficient ($K_f$) thus reducing the area available for filtration per glomerulus. It is clear that following ureteral obstruction there is a reduction in $K_f$ (Ichikawa I 1985), but there is no evidence for an alteration in the intrarenal distribution of cortical blood flow after 24 hours of UUO in the rat (Hsu CH 1977). Alternatively a reduction in filtration fraction could result from preferential constriction of the preglomerular blood vessels, as this would lower both blood flow and glomerular capillary pressure, thus resulting in a greater decrement in glomerular filtration rate than blood flow.

The mechanism responsible for the second phase of progressive vasoconstriction appears in part to be an augmented production of thromboxane $A_2$. The chronically obstructed rabbit kidney displays an enhanced ability to metabolize arachidonic acid (Okegawa T 1983), and thromboxane synthetase activity is increased (Morrison AR 1977). Thromboxane $A_2$ also causes a reduction in $K_f$ (Mene P 1986). Inhibition of thromboxane synthetase dramatically improves post-obstructive renal haemodynamics and reverses the renal vasoconstriction of acute ureteral obstruction.
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[Yarger WE 1980, Ichikawa I 1985], and increases $K_{f}$ [Ichikawa I 1985]. The observation of a tight negative correlation between the excretion rate of thromboxane $B_2$ and both glomerular filtration rate and renal plasma flow in this model suggests that increased intrarenal production of thromboxane $A_2$ may contribute to the pathophysiology of the altered haemodynamic which are seen following ureteral obstruction.

It is also of note that the production of the vasodilator prostaglandin $E_2$ also increases progressively with the duration of ureteral obstruction. Paradoxically a significant correlation was also observed between the excretion of this vasodilator prostanoid and renal haemodynamic parameters. Whilst such a change may be expected to partially antagonise the vasoconstrictor response seen as a result of enhanced thromboxane production, the net balance of vasoconstrictor to vasodilator forces is clearly in the direction of vasoconstriction in this model.

The mechanism of the diuresis and natriuresis observed following the release of ureteral obstruction is complex. This study confirms previously reported differences between unilateral and bilateral ureteral obstruction [Klahr S 1986]. A post-obstructive diuresis and natriuresis is only seen following the release of more than 12 hours of bilateral ureteral obstruction. No diuresis or natriuresis was seen following the release of unilateral ureteral obstruction in the current studies, although altered water and sodium excretion by the post-obstructed kidney can be unmasked by volume expansion [Wilson DR 1974, Harris KPG 1991].

Following ureteral obstruction an alteration in the handling of sodium reabsorption by proximal and distal tubule has been reported [Yarger WE 1972, Buerkert J 1976, Beukert 1977, McDougal WS 1972]. In addition there is an increase
in the fraction of filtered water delivered to the early distal tubule [Yarger WE 1972, Buerkert J 1976], suggesting that there is a decreased ability of the thick ascending limb of Henle's loop to resorb salt. Prostaglandin E₂ is known to inhibit chloride transport in the thick ascending limb [Higashihara E 1979, Stokes JB 1979]. This observation taken together with the enhanced ability of the obstructed kidney to produce prostaglandin E₂ reported in this and other studies [Okegawa T 1983, Lefkowith 1984], suggests that prostaglandin E₂ could have a role in the development of post-obstructive diuresis and natriuresis. The strong correlation between prostaglandin E₂ excretion and parameters of diuresis and natriuresis would be consistent with such a causal role. Against this, experimental studies in humans have suggested that prostaglandin E₂ excretion increases simply as a result of increases in urine flow and an increase may not therefore represent increased intrarenal synthesis [Lifschitz MD 1986]. If true this would imply that enhanced urinary prostaglandin E₂ excretion could result from an increased urine flow rather than be a cause of diuresis per se. However in the rat urine flow rate and prostaglandin excretion do not appear to be necessarily linked and an increase in prostaglandin E₂ excretion more accurately mirrors intrarenal synthesis [Fejes-Toth 1983].
CHAPTER 5

CHARACTERISATION AND KINETICS OF THE IMMUNE CELL INFILTRATE IN THE KIDNEY FOLLOWING URETERAL OBSTRUCTION

5.1 Introduction

More than twenty years ago, chronic renal obstruction in rabbits was found to be associated with a proliferation of interstitial fibroblasts and an infiltration of mononuclear cells [Nagle RB 1973, Nagle RB 1976]. These changes have now been linked to the increase in prostaglandin E₂ production by the chronically hydronephrotic rabbit kidney [Davis BB 1983] and it has been postulated that the infiltration of the renal parenchyma by mononuclear cells may affect the augmented release of thromboxane A₂ and prostaglandin E₂ in response to bradykinin [Okegewa T 1983] or endotoxin [Lefkowith JB 1984]. The leukocyte infiltrate in these lesions had not been previously characterized. In addition, the studies utilized kidneys that had been obstructed 3-10 days or longer with severe structural damage, histological evidence of fibrosis, and irreversible loss of function. Therefore the current studies were designed to define the initial phases of cell infiltration in a model of acute and reversible ureteral obstruction in which there is no necrosis and in which renal functional abnormalities are transient [Klahr S 1983].

The hypothesis that the cellular infiltrate may play a role in the alteration of renal function following ureteral obstruction is examined. The kinetics of the influx of cells are correlated with the changes in renal haemodynamics and the alterations in renal prostanoid production which were described in Chapter 4.
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5.2 Methods.

5.2.1 Analysis of Immune Cell Infiltrates.

Four different experimental groups of animals were employed in order to characterise the immune cell infiltrate in i) the interstitium, and ii) the glomerulus in response to ureteral obstruction.

1. Control animals.
2. Animals which had undergone bilateral ureteral obstruction
3. Animals which had undergone unilateral ureteral obstruction
4. Animals which had undergone ureteral obstruction which had subsequently been released.

The number of animals used in each experiment is indicated in the text or in the legends to the tables and figures.

After varying durations of ureteral obstruction (0, 4, 12 and 24 hours) the animals were anaesthetized, the kidneys perfused in situ and then removed. The animal was subsequently killed by exsanguination while under deep anaesthesia. The kidneys were then processed as described in Chapter 3 in order to enumerate the leukocyte content of the interstitium and glomeruli. Animals were also studied 2 and 6 days following the release of ureteral obstruction.

Immunohistochemical staining was also carried out on the sections in order to determine the position of the infiltrating cells relative to other renal structures.
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5.3 Results.

5.3.1 Quantification of Interstitial Leukocytes following Ureteral Obstruction.

Adult rats underwent bilateral ureteral obstruction as previously described. Four groups of rats were studied to determine the kinetics and localization of the obstructed infiltrate at 4, 12 and 24 hours after obstruction. These were the same time points used for the studies of renal function. Three rats per time point were studied.

An increase in cells expressing the leukocyte common antigen was apparent in both cortex and medulla as early as 4 hours (Figure 5.1a and b). The infiltrate appeared to plateau at 12 hours in the cortex, but increased up to 24 hours in the medulla. On a per weight basis, the cortex experienced a somewhat greater infiltrate than did the medulla.
Figure 5.1a  The effect of bilateral ureteral obstruction on the number of cells expressing the leukocyte common antigen in kidneys at timed intervals after obstruction. LC+ are cells staining positive for the leukocyte common antigen (OX-1). The results represent the mean ± sem of single kidneys of three rats. Representative portions of cortex were dissected out, weighed, and subjected to enzymatic digestion and labelled. The control kidneys were taken from littermates that did not undergo any procedure.
**Figure 5.1b** The effect of bilateral ureteral obstruction on the number of cells expressing the leukocyte common antigen in kidneys at timed intervals after obstruction. LC⁺ are cells staining positive for the leukocyte common antigen (OX-1). The results represent the mean ± sem of single kidneys of three rats. Representative portions of medulla were dissected out, weighed, and subjected to enzymatic digestion and labelled. The control kidneys were taken from littermates that did not undergo any procedure.
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5.3.2 Localisation of the Infiltrate following Ureteral Obstruction.

Histological analysis of a kidney from a rat that had bilateral ureteral obstruction for 24 hours revealed the presence of a non-destructive mononuclear cell infiltrate. There were no neutrophils. It is of note that the infiltrate was inconspicuous in the obstructed kidneys if they had not been stained specifically for leukocytes. However, immunohistochemical labelling of the leukocytes revealed numerous leukocytes in a very distinctive peri-tubular ring pattern. There appeared to be preferential localization around epithelium of the distal tubule in the obstructed kidney (Figures 5.2a and 5.2b). Immunohistochemical labelling of kidneys which had not previously been obstructed typically revealed the presence of only 1-2 leukocytes per high powered field.
Figure 5.2a Immunostaining of sections of obstructed kidneys for leukocytes. Kidney sections were prepared following 24 hours of bilateral ureteral obstruction. Renal peri-tubular localization of cells positively labelled for leukocyte antigen after 24 hours of acute ureteral obstruction is seen. The figure shows cortical distal tubule, x 600.
Figure 5.2b  **Immunostaining of sections of obstructed kidneys for leukocytes.**
Kidney sections were prepared following 24 hours of bilateral ureteral obstruction. Renal peri-tubular localization of cells positively labelled for leukocyte antigen after 24 hours of acute ureteral obstruction is seen. The figure shows medulla, x 600.
5.3.3 Immune cell subset analysis.

Further delineation of the leukocyte subsets of the obstruction-induced infiltrate was carried out at 24 hours, the time of maximum infiltration. The results of three experiments, utilizing duplicate animals in each experiment are presented in Table 5.1.

Table 5.1 Immune Cell Subsets in Acute Renal Obstruction. (10^6 cells/gram tissue).

<table>
<thead>
<tr>
<th></th>
<th>MΦ</th>
<th>T_h</th>
<th>T_s</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cortex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.2±0.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Obstructed</td>
<td>48.5±10.8</td>
<td>2.4±0.7</td>
<td>11.0±2.2</td>
<td>0</td>
</tr>
<tr>
<td><strong>Medulla</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.5±0.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Obstructed</td>
<td>8.9±1.8</td>
<td>1.0±0.7</td>
<td>3.9±1.0</td>
<td>0</td>
</tr>
</tbody>
</table>

Duplicate rats underwent 24 hours of bilateral ureteral obstruction. After sacrifice, portions of cortex and medulla were dissected free, enzymatically digested and labelled for cells expressing antigen markers characteristic of macrophages (M), T lymphocytes of the helper subset (T_h), T lymphocytes of the suppressor subset (T_s), and B lymphocytes (B). Each point represents the mean ± sem of 3 groups of paired rats, totalling 6 kidneys per point.

A small population of macrophages was noted in the cortex of control, unobstructed rats. Post-obstruction, there was a 15-fold increase. Thus, macrophages appear to constitute the predominant cell population in the acutely obstructed kidney. Increases in both a suppressor cell and helper cell subpopulation of T lymphocytes became apparent after 24 hours of obstruction. However, there was a preferential infiltration of T suppressor lymphocytes when compared to T helper cells. B lymphocytes were not seen either in control or in obstructed kidneys. The same cell
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types predominated in the medulla with an approximately 15-fold increase in macrophages following obstruction. The number of medullary macrophages in both control and obstructed kidneys were lower than that observed in the cortex. A preferential infiltration of T lymphocytes of the suppressor cell subset was also observed, although the difference was less marked than that seen in the cortex. Neither B lymphocytes nor neutrophils were observed in the medulla.

5.3.4 Unilateral vs Bilateral Ureteral Obstruction.

Leukocyte infiltration in unilateral obstructed kidneys, together with the contralateral control (non-obstructed) kidney was compared to that observed in bilateral obstructed kidneys at the time of maximum infiltration. As shown in Table 5.2, leukocyte infiltration in the two models was comparable.

Table 5.2 Renal Leukocyte Content: comparison between Unilateral and Bilateral Ureteral Obstruction.

<table>
<thead>
<tr>
<th></th>
<th>Control (no obstruction)</th>
<th>Bilateral obstruction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (unilateral obstruction)</td>
<td>3.2</td>
<td>24.0</td>
</tr>
<tr>
<td>Unilateral obstruction</td>
<td>3.6</td>
<td>26.2</td>
</tr>
</tbody>
</table>

Pairs of rats underwent bilateral or unilateral ureteral obstruction for 24 hours. A cross-section of each kidney was then digested and the cells expressing the leukocyte common antigen enumerated. The results represent the average counts of two kidneys from duplicate rats. Control kidneys were taken from littermates undergoing no procedure or from the contralateral, non-obstructed kidney in the model of unilateral obstruction.
5.3.5 The Effect of Obstruction on Leukocyte content of Glomeruli.

The representation of the resident mesangial macrophages in glomeruli taken from normal animals with unobstructed kidneys, and from both the obstructed and contralateral control kidneys of rats with unilateral ureteral obstruction of 24 hours duration was examined. As shown in Figure 5.3, compared to both control kidneys and the paired contralateral non-obstructed kidney, glomeruli from the obstructed kidneys of animals with unilateral ureteral obstruction showed a significant depletion of resident macrophages 24 hours after the onset of obstruction, a time of increasing macrophage migration into the interstitium.
Figure 5.3 The macrophage content of glomeruli following ureteral obstruction. The glomeruli were isolated from kidneys with no obstruction (control), unilaterally obstructed kidneys (obstructed, UUO), and the paired, contralateral, non-obstructed kidney (control, UUO). Results represent the mean counts ± sem of 50 glomeruli from duplicate kidneys. The duration of the unilateral obstruction was 24 hours.
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5.3.6 The Effect of release of Obstruction on the Leukocyte content of the Interstitium (Recovery Phase).

The effect of releasing obstruction on the leukocyte content of both the cortex and medulla of the kidney was examined. The unilateral obstruction model was used in order to utilize the paired, contralateral, unobstructed kidney as a control. Reversible ureteral obstruction was achieved using the methods outlined in Chapter 3.

Leukocytes in the obstructed kidney were quantified at the time of release of 24 hours of obstruction (day 1), and at 2 days and at 6 days after the release of obstruction (days 3 and 7, respectively) and compared to the leukocyte content of the contralateral unobstructed kidney in the same animal. The infiltrating patterns of macrophages and T lymphocytes in the cortex are compared in Figure 5.4a. Two days after the release of obstruction (day 3), the macrophage content of the cortical interstitium appeared to increase modestly and then to decline to near normal levels by 6 days after the release of obstruction (day 7). In contrast, the T lymphocytes in the cortex rapidly diminished as soon as the obstruction was released to less than 20% of their obstructed values at 2 days after release of obstruction, with a further small decrement noted 4 days later. Of potential interest is the fact that a small increase in both cell populations was noted as long as a week after the release of obstruction.

In the medulla, the macrophage population declined promptly after the release of obstruction, as did the T lymphocytes (Figure 5.4b). As was seen in the cortex, the leukocyte infiltrate was largely reversible after the release of obstruction, but a detectable increment in both macrophages and T lymphocytes was measurable as late as 6 days after the obstructive event. In contrast, two days after the release of
obstruction, the number of glomerular macrophages had increased to slightly above that seen in control glomeruli (Figure 5.5). Moreover, glomerular macrophages continued to accumulate to greater than double the normal amount seen in the contralateral kidney 6 days after the release of obstruction. This was associated with a modest increase in glomerular T lymphocytes over the same time course.
Figure 5.4a The number of macrophages and T lymphocytes in the kidney after acute obstruction followed by release of the obstruction. Pairs of rats were sacrificed after 24 hours of obstruction, and at 2 days and 6 days after release of the obstruction. The control measurements at day 0 were obtained from kidneys of littermates. ○ = macrophages, ● = T cells (pan T cell marker OX 19). The shaded box represents the duration of ureteral obstruction.
Figure 5.4b  The number of macrophages and T lymphocytes in the kidney after acute obstruction followed by release of the obstruction. Pairs of rats were sacrificed after 24 hours of obstruction, and at 2 days and 6 days after release of the obstruction. The control measurements at day 0 were obtained from kidneys of littermates. O = macrophages, • = T cells (pan T cell marker OX 19). The shaded box represents the duration of ureteral obstruction.
Figure 5.5  The glomerular content of macrophages and T lymphocytes after 24 hours of ureteral obstruction followed by release of the obstruction. The results reflect the mean ± sem of 50 glomeruli counted per kidney of duplicate rats sacrificed after 24 hours of obstruction (day 1) and at 2 days and 6 days after the release of the obstruction (days 3 and 7 post ureteral obstruction respectively). The mean number of cells ± sem of the contralateral control kidney after 24 hours of obstruction was used to provide control data.
5.4 Discussion.

These observations indicate that an immune cell infiltrate comprises one of the earliest renal responses to acute ureteral obstruction. Although the infiltrate is observed within 4 hours, peak response appears to occur at 12 hours, after which a plateau is observed. The leukocytes do not compose a destructive infiltrate. Instead they appear to form distinctive rings around the tubules, particularly distal tubules. In normal, unobstructed kidneys, both glomeruli and cortex are characterized by the presence of small numbers of resident leukocytes that are predominantly macrophages. The normal medulla is almost completely devoid of resident leukocytes, presumably secondary to the hypertonic environment. In obstruction, however, the medulla is also invaded by mononuclear cells to an extent comparable to that of the cortex. Unexpectedly, the leukocyte invasion of the interstitium in the obstructed kidney is associated with a relative depletion of resident leukocytes from the glomeruli. Although no information is available as to the destination of the glomerular leukocytes, which are resident mesangial macrophages [Schreiner GF 1981], one possibility is that they are induced to migrate from the glomerulus to the interstitium via the mesangial stalk in response to signals from the obstructed interstitium. A similar migration has been observed in a model of lupus nephritis [Kimura M 1987].

The mononuclear cell infiltrate consists predominantly of macrophages. The second major leukocyte population consists of T lymphocytes of the cytotoxic, suppressor cell subclass. Some degree of selectivity is implied in the observation that T lymphocytes of the helper type do not form a significant component of the infiltrating cells, despite the fact that they predominate in the peripheral circulation [Mampaso F 1983]. B lymphocytes do not appear to play a role in the phenomenon.
CHAPTER 5 CHARACTERISATION OF INFILTRATE

The inference that the renal infiltrate of acute ureteral obstruction is not destructive is supported by the absence of neutrophils in this lesion.

The infiltration is slowly reversible, requiring several days after the release of obstruction to revert to near normal levels. An interesting exception lies in the data on glomerular repopulation with resident macrophages. After release of the obstruction, glomerular macrophages accumulate to a level significantly increased when compared to control glomeruli. The increase is comparable to the degree of macrophage infiltration observed in a model of moderate glomerulonephritis induced by nephrotoxic serum [Schreiner GF 1984]. It is interesting to speculate that this post-obstructive macrophage infiltration may constitute one of the explanations for the loss of functional glomeruli previously observed in the post-obstructed kidney of the rat [Bander SJ 1985]. Whether the mild increase in glomerular lymphocytes occurring at this time functionally contributes to the evolution of a glomerular lesion is unknown.

The kinetics of the macrophage and leukocyte invasion temporally parallels the decline in glomerular filtration reported in Chapter 4. The decline in glomerular filtration following ureteric obstruction as previously discussed is due to a net decrease in the transglomerular pressure and a decrease in $K_f$, the glomerular ultrafiltration coefficient [Wright F 1982, Blantz RC 1975]. Initially following ureteral obstruction there is an initial prostaglandin-dependent increase in renal blood flow [Allen JT 1978, Klahr S 1983, Wright FS 1982, ], which declines after 4 hours at the time when the leukocyte infiltrate is becoming prominent. It subsequently reaches 40-70% of normal values by 24 hours, due to progressive renal vasoconstriction. The mechanism responsible for the second phase of progressive
vasoconstriction appears in part to be an augmented production of thromboxane $A_2$ which at the same time can also cause a reduction in $K_f$ [Mene P 1986]. The chronically obstructed rabbit kidney displays an enhanced ability to metabolize arachidonic acid [Okegewa T 1983], and thromboxane synthetase activity is increased [Morrison AR 1977]. Inhibition of thromboxane synthetase dramatically improves post-obstructive renal haemodynamics and reverses the renal vasoconstriction of acute ureteral obstruction [Yarger WE 1980, Ichikawa I 1985], and increases $K_f$ [Ichikawa I 1985].

As shown in Chapter 4 Thromboxane $B_2$ excretion increases with time following obstruction as renal function is declining. There is a close temporal relationship between the macrophage infiltrate, the increase in thromboxane production, and the decline in renal function; this is consistent with, but does not prove, a causal relation between these parameters in acute obstruction. Nonetheless, macrophages are a potent source of thromboxane [Halushka PV 1981].

In summary ureteral obstruction is associated with the release of a factor or factors that acutely induce macrophages and lymphocytes to infiltrate the kidney. The leukocytes may promote, or directly contribute to, a locally enhanced production of thromboxane $A_2$. These vasoactive substances may contribute to the alterations in renal haemodynamics that are seen following ureteral obstruction. Whether other substances or factors released by macrophages modulate epithelial cell function remains unknown. What contribution, if any, the accompanying suppressor lymphocytes in this lesion make to the renal response to obstruction requires further characterisation.

The nature of the stimulus coupling obstruction of the urinary tract to the
leukocyte infiltration is further investigated in Chapter 8.
CHAPTER 6

THE EFFECT OF LEUKOCYTE DEPLETION ON THE
FUNCTION OF THE POST-OBSTRUCTED KIDNEY.

6.1 Introduction

Chapter 5 identified that following ureteral obstruction there is an influx of leukocytes, predominantly macrophages, into the cortex and medulla of the kidney which peaks at about 12-24 hours after the onset of obstruction. Chronic obstruction is known to be associated with increased interstitial cellularity and enhanced production of arachidonate metabolites by the obstructed kidney [Nagle RB 1973, Nagle RB 1976, Okegawa T 1983]. The activity of thromboxane synthetase is increased in the obstructed kidney [Morrison AR 1977, Morrison AR 1978], and obstructed kidneys show an increase in cyclo-oxygenase activity [Currie MG 1981]. In the rabbit pretreatment with nitrogen mustard, so as to induce leukopaenia blocks peptide stimulated arachidonate metabolism and selectively prevents the increase in thromboxane synthetase activity in the chronically obstructed kidney [Lefkowith JB 1984]. Thus, the infiltration of leukocytes may be a critical determinant of the changes in arachidonate metabolism seen after obstruction. Indeed macrophages have been shown to produce a variety of arachidonic acid metabolites in vitro [Scott WA 1980, Halushka PV 1981].

The kinetics of the macrophage influx into the obstructed kidney closely parallel the altered arachidonate metabolism and the accompanying decline in glomerular filtration rate (see Chapter 5). The vasoconstrictor thromboxane A₂ has been implicated in the pathogenesis of this state since specific inhibitors of thromboxane synthesis has been shown to improve renal haemodynamics in the post-
obstructed kidney [Yarger WE 1980, Ichikawa I 1985]. It is, therefore, possible that infiltration of the renal parenchyma by leukocytes may directly mediate some of the changes in kidney function which occur with ureteral obstruction, via the production of vasoactive arachidonate metabolites or other as yet undefined products. The present studies were designed to examine the role of leukocyte infiltration on renal function in the post-obstructive period.

6.2 Methods.

Bilateral ureteral obstruction was produced as described previously in Chapter 3, and were studied 24 hours later. Twenty-four hours prior to induction of bilateral ureteral obstruction (48 hours prior to clearance studies or leukocyte counts in the kidney) the experimental group of rats received whole body irradiation of 1315 rads over 10 minutes (Gamma cell 40, Atomic Energy of Canada Ltd.). It has been previously shown that this dose of irradiation, within the time course studied, effectively depletes the kidney of resident interstitial leukocytes and inhibits the influx of macrophages in experimental nephrotoxic serum glomerulonephritis [Fradet Y 1980, Schreiner GF 1978]. Rats from each group either had clearance studies or had their kidneys analyzed for leukocyte content.

In order to assess the effect of irradiation on the renal function of normal animals, a further group of rats underwent a sham laparotomy with ureteric manipulation but no ureteral obstruction 24 hours prior to study. Half of these sham operated control rats had undergone whole body irradiation 24 hours prior to the sham laparotomy. Food, but not water was withheld from the time of the surgery.
6.2.1 Peripheral White Cell Count.

In order to examine the effect of the irradiation on peripheral white cell number, total and differential white blood cell counts were measured in blood obtained from 3 normal and 6 rats which had been irradiated 48 hours previously. (see Appendix 5 for method).

6.2.2 Leukocyte Content of the Renal Parenchyma.

Eight rats underwent bilateral ureteral obstruction, 4 having been irradiated 24 hour earlier. After 24 hours of bilateral ureteral obstruction the animals were anaesthetized and the kidneys were perfused and prepared for interstitial leukocyte labelling as described in Chapter 3. An additional three rats which had not undergone ureteric obstruction had the kidneys prepared in a similar way for leukocyte content analysis.

6.2.3 Clearance Studies.

Sixteen rats underwent bilateral ureteral obstruction with 8 having been irradiated 24 hours earlier. Twenty-four hours after bilateral ureteral obstruction the animals were prepared for clearance studies as described in Chapter 3. A sample of urine was collected on ice and stored at -70° C for subsequent thromboxane B₂ and prostaglandin E₂ determination in 6 animals from each group.

An additional 11 rats were used as non-obstructed controls (sham operated), 6 of which had been irradiated 48 hours earlier in order to assess the effect of irradiation alone on renal function. These rats were subsequently prepared for clearance studies as described above, except one non-obstructed ureter was cannulated for the collection of urine (urine from the contralateral kidney was voided via the bladder and was not collected). A sample of urine was saved for thromboxane B₂
determination. (Urine was not analyzed for thromboxane B\textsubscript{2} if the sample was bloody.)

6.3 Results.

6.3.1 Peripheral White Cell Count.

The effect of 48 hours prior irradiation on peripheral and differential white cell counts is shown in Table 6.1. Under control conditions the majority of circulating leukocytes in the rat are lymphocytes. As expected the irradiation protocol produced a highly significant fall in total white cell count; lymphocytes and monocytes were largely eliminated from the circulation with neutrophil number remaining unchanged.

6.3.2 Leukocyte Content of the Renal Parenchyma.

The leukocyte content of the cortex and medulla of kidneys from normal animals (controls) and from kidneys of rats with ureteral obstruction which had either previously been irradiated or not is shown in Figure 6.1. As previously shown in Chapter 5 bilateral ureteric obstruction resulted in a dramatic and highly significant (P < 0.001) influx of leukocytes into both the cortex and medulla. This was abolished by irradiation such that leukocyte content after bilateral obstruction in rats previously irradiated was similar to that seen in normal kidneys (controls).
### Table 6.1 The Effect of Irradiation 48 hours previously on Total and Differential White Cell Counts

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Irradiated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total WCC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>x10^9/l</td>
<td>8.33±1.65</td>
<td>1.37±0.20*</td>
</tr>
<tr>
<td><strong>Lymphocytes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>x10^9/l</td>
<td>6.44±0.92</td>
<td>0.06±0.03*</td>
</tr>
<tr>
<td>(%)</td>
<td>(79.3±5.5)</td>
<td>(3.7±1.5)</td>
</tr>
<tr>
<td><strong>Neutrophils</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>x10^9/l</td>
<td>0.94±0.29</td>
<td>1.27±0.19</td>
</tr>
<tr>
<td>(%)</td>
<td>(10.7±1.9)</td>
<td>(93.5±2.4)</td>
</tr>
<tr>
<td><strong>Monocytes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>x10^9/l</td>
<td>0.35±0.26</td>
<td>0.03±0.02</td>
</tr>
<tr>
<td>(%)</td>
<td>(4.0±2.3)</td>
<td>(1.7±1.1)</td>
</tr>
<tr>
<td><strong>Eosinophils</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>x10^9/l</td>
<td>0.03±0.03</td>
<td>0.01±0.01</td>
</tr>
<tr>
<td>(%)</td>
<td>(0.3±0.3)</td>
<td>(0.8±0.8)</td>
</tr>
<tr>
<td><strong>Basophils</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>x10^9/l</td>
<td>0.22±0.22</td>
<td>0</td>
</tr>
<tr>
<td>(%)</td>
<td>(2.3±2.3)</td>
<td>0</td>
</tr>
</tbody>
</table>

* P < 0.001 Irradiated vs Control
Figure 6.1a  The effect of irradiation on the leukocyte content of the kidney following ureteral obstruction. The number of cells expressing the leukocyte antigen in the cortex of normal kidneys (control) or kidneys after 24 hours of bilateral ureteral obstruction (BUO non-irradiated) and BUO with prior irradiation (BUO irradiated). BUO increased the leukocyte content of the cortex (P < 0.001) as previously described. Prior irradiation completely prevented the increase in leukocyte content following BUO.
**Figure 6.1b** The effect of irradiation on the leukocyte content of the kidney following ureteral obstruction. The number of cells expressing the leukocyte antigen in the medulla of normal kidneys (control) or kidneys after 24 hours of bilateral ureteral obstruction (BUO non-irradiated) and BUO with prior irradiation (BUO irradiated). BUO increased the leukocyte content of the medulla ($P < 0.001$) as previously described. Prior irradiation completely prevented the increase in leukocyte content following BUO.
6.3.3 Clearance Studies.

There was no statistical significant difference between the weights of the non-irradiated and irradiated rats prior to obstruction (252.0 ± 4.2 vs 240.8 ± 6.5 g). After obstruction the non-irradiated and irradiated animals lost a comparable amount of weight (9.8 ± 0.9 vs 8.5 ± 1.8 g in 24 hours), which represented approximately a 4% weight loss in each group. The weight of the non-irradiated and irradiated non-obstructed controls was similar (269.5 ± 8.35 vs 272.5 ± 10.0 N.S.) but following sham laparotomy without ureteral obstruction the weight loss was approximately 6.5% in each group.

At the time of study there was no difference in the blood urea nitrogen level of the non-irradiated or irradiated obstructed animals (129.3 ± 6.3 vs 124.1 ± 6.3 mg/dl) or in the haematocrit (41.0 ± 0.9 vs 39.5 ± 0.8%). However the haematocrit of the animals with ureteral obstruction was significantly lower (P < 0.002) than that in either non-irradiated or irradiated control animals (45.2 ± 0.3% and 44.7 ± 0.7%).

The results for \( C_a \) and \( C_{pH} \) following unilateral release of bilateral ureteral obstruction are shown in Figure 6.2a and Figure 6.2b. Bilateral ureteral obstruction resulted in a highly significant fall in \( C_a \) to 30% of control values (1.58 ± 0.12 vs 5.34 ± 0.1 ml/min/kg BW P < 0.001). Irradiation of the animals prior to induction of bilateral ureteric obstruction resulted in significantly higher values for \( C_a \) of the post-obstructed kidney (2.97 ± 0.15 vs 1.58 ± 0.12 ml/min/Kg BW P < 0.001) which was 61% of values from control irradiated animals. Prior irradiation had a similar effect on \( C_{pH} \) of the obstructed kidney and as a result filtration fraction was unchanged (0.23 ± 0.02 vs 0.22 ± 0.01 N.S.).
Figure 6.2a The effect of prior irradiation on inulin clearances ($C_{in}$) in rats 24 hours after a sham laparotomy (control) or 3 hours after unilateral release of 24 hours of BUO. The results are expressed as mean ± sem. The results from the animals who had a sham laparotomy are from a single kidney. Irradiation had no significant (N.S.) effect on the renal function of rats after a sham laparotomy (control), but significantly improved the renal function of the rats with 24 hours of ureteral obstruction (BUO).
Figure 6.2b The effect of prior irradiation on PAH clearances ($C_{PAH}$) in rats 24 hours after a sham laparotomy (control) or 3 hours after unilateral release of 24 hours of BUO. The results are expressed as mean ± sem. The results from the animals who had a sham laparotomy are from a single kidney. Irradiation had no significant (N.S.) effect on the renal function of rats after a sham laparotomy (control), but significantly improved the renal function of the rats with 24 hours of ureteral obstruction (BUO).
It is of note that even with irradiation prior to bilateral ureteric obstruction, the renal function of the post-obstructed kidney remained depressed below values obtained for one non-obstructed kidney in the control rats (2.97 ± 0.15 vs 4.81 ± 0.40 ml/min/Kg BW P < 0.001 for $C_u$ and 13.99 ± 0.94 vs 20.88 ± 2.51 ml/min/Kg BW P < 0.002 for $C_{\text{TAU}}$).

The results for urine flow, fractional water excretion and absolute and fractional sodium excretion are summarized in Table 6.2 for non-irradiated and irradiated control rats and for non-irradiated and irradiated obstructed rats. After unilateral release of bilateral ureteral obstruction the animals exhibited a marked post-obstructive diuresis and natriuresis when compared to controls, with significantly higher absolute and fractional excretions of water and sodium. Prior irradiation of the animals decreased fractional water and sodium excretion significantly, although not to control values. In contrast in normal animals irradiation did not affect any of the measured parameters of renal function.
### Table 6.2
Tubular function in control rats and following unilateral release of 24 hours of BUO with and without prior irradiation.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>BUO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-irradiated</td>
<td>Non-irradiated</td>
</tr>
<tr>
<td></td>
<td>(n=5)</td>
<td>(n=8)</td>
</tr>
<tr>
<td>Urine flow (µl/min)</td>
<td>18.3±2.2</td>
<td>43.5±4.6*</td>
</tr>
<tr>
<td></td>
<td>17.8±2.3</td>
<td>56.8±3.2**</td>
</tr>
<tr>
<td>Fractional water excretion (%)</td>
<td>1.4±0.2</td>
<td>1.5±0.1</td>
</tr>
<tr>
<td>Sodium excretion (µmol/min)</td>
<td>1.7±0.2</td>
<td>1.0±0.3</td>
</tr>
<tr>
<td></td>
<td>11.4±0.9*</td>
<td>8.5±0.8*</td>
</tr>
<tr>
<td></td>
<td>5.2±0.4*</td>
<td>5.2±0.6**</td>
</tr>
<tr>
<td>Fractional sodium excretion (%)</td>
<td>0.9±0.1</td>
<td>0.5±0.2</td>
</tr>
<tr>
<td></td>
<td>7.8±0.8*</td>
<td>5.2±0.6**</td>
</tr>
</tbody>
</table>

* P < 0.001 of sham operated control

^ P < 0.01 of Non-irradiated BUO.
CHAPTER 6 LEUKOCYTE DEPLETION

Figure 6.3 shows the thromboxane excretion expressed in pg/min for both non-irradiated and irradiated control animals and non-irradiated and irradiated animals with unilateral release of bilateral ureteral obstruction. It is clear that after release of bilateral ureteric obstruction there is a significant rise in thromboxane B2 excretion which is significantly reduced by prior irradiation. Even with irradiation prior to unilateral release of bilateral ureteral obstruction thromboxane B2 excretion remained significantly higher than control values (19.03 ± 1.94 vs 9.92 ± 1.73 p<0.05). Irradiation had no significant effect on the thromboxane B2 excretion in control rats.

Within this cohort of experimental animals (BUO vs sham operated, irradiated vs non-irradiated) a significant correlation between thromboxane B2 excretion and both Cw and CpAR (Figure 6.4a and b) was again observed.

The data for thromboxane B2 and prostaglandin E2 excretion in the urine after unilateral release of bilateral ureteric obstruction are summarized in Table 6.3. The irradiated animals excreted significantly less thromboxane B2 in the urine whether the data are expressed in absolute terms or when corrected per unit of Cw or CpAR. Prostaglandin E2 excretion by the obstructed kidney of irradiated rats was reduced compared to the values of non-irradiated animals, but this difference did not reach statistical significance because of the large scatter of values. A significant correlation was observed between absolute prostaglandin E2 excretion and fractional water excretion (r = 0.67, P < 0.02) and fractional sodium excretion (r = 0.8, P < 0.002) in the current set of experiments comparing the irradiated and non irradiated obstructed animals.
Figure 6.3  Urinary excretion of thromboxane B$_2$ following ureteral obstruction. Data is shown from sham operated non-obstructed rats (control) and in rats 3 hours after unilateral release of 24 hours of bilateral ureteral obstruction (BUO) with and without prior irradiation. The excretion rates from the sham operated rats are from a single kidney. Irradiation had no effect on thromboxane B$_2$ excretion in sham operated rats, but reduced it in rats with 24 hours of ureteral obstruction.
Figure 6.4a Relationship between thromboxane B₂ excretion and inulin clearances (Cᵢₙ). There is a strong and significant correlation between thromboxane B₂ excretion rates and inulin clearances in the various study conditions (control ± irradiation and BUO ± irradiation). This supports the hypothesis that the generation of thromboxane A₂ in the kidney by infiltrating leukocytes is responsible for the altered haemodynamics seen following ureteral obstruction.
Figure 6.4b Relationship between thromboxane B₂ excretion and PAH clearances ($C_{PAH}$). There is a strong and significant correlation between thromboxane B₂ excretion rates and PAH clearances in the various study conditions (control ± irradiation and BUO ± irradiation). This supports the hypothesis that the generation of thromboxane A₂ in the kidney by infiltrating leukocytes is responsible for the altered haemodynamics seen following ureteral obstruction.
### Table 6.3 Effect of irradiation on thromboxane B₂ and PGE₂ excretion in the urine after unilateral release of bilateral ureteral obstruction.

<table>
<thead>
<tr>
<th></th>
<th>Non-Irradiated (n=6)</th>
<th>Irradiated (n=6)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Thromboxane B₂</strong> (pg/min)</td>
<td>32.5±5.0</td>
<td>19.0±1.9</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td><strong>Thromboxane B₂</strong> (pg/min/ml Cᵥ)</td>
<td>81.6±11.7</td>
<td>29.6±3.6</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>Thromboxane B₂</strong> (pg/min/ml Cᵥ)</td>
<td>15.8±1.8</td>
<td>6.9±1.1</td>
<td>&lt; 0.002</td>
</tr>
<tr>
<td><strong>PGE₂</strong> (pg/min)</td>
<td>133.8±29.21</td>
<td>110.8±44.0</td>
<td>NS</td>
</tr>
<tr>
<td><strong>PGE₂</strong> (pg/min/ml Cᵥ)</td>
<td>352.9±68.7</td>
<td>175.8±73.9</td>
<td>NS</td>
</tr>
<tr>
<td><strong>PGE₂</strong> (pg/min/ml Cᵥ)</td>
<td>72.8±18.0</td>
<td>45.3±19.7</td>
<td>NS</td>
</tr>
</tbody>
</table>
CHAPTER 6 LEUKOCYTE DEPLETION

6.4 Discussion.

The pathophysiology of the haemodynamic alterations following ureteral obstruction have been previously discussed. The results presented here, confirm that there is a marked fall in both glomerular filtration rate and renal plasma flow following bilateral ureteral obstruction. This is associated with an increase in urinary excretion of thromboxane B$_2$, which reflects increased intrarenal synthesis of thromboxane A$_2$. Leukocytes, predominantly macrophages, infiltrate the kidney in large numbers within 12 hours of bilateral ureteral obstruction in the rat and this infiltration is associated with enhanced thromboxane B$_2$ excretion in the urine and a decline in glomerular filtration rate (Chapters 4 and 5). Elimination of the infiltrate by prior irradiation of the animals reduces thromboxane B$_2$ excretion and at the same time there is a significant improvement in renal haemodynamics of the post-obstructed kidney. A causal role for intrarenal thromboxane A$_2$ generation in the decline in renal function following ureteral obstruction is supported by the observation that renal function correlates significantly with the renal excretion of thromboxane B$_2$ in these studies.

These studies provide the first demonstration that infiltrating leukocytes contribute to haemodynamic changes in the post-obstructive kidney and suggest that the leukocyte infiltrate is in part responsible for the decline in glomerular filtration rate and renal plasma flow seen after obstruction possibly via the production of vasoactive prostanoids such as thromboxane A$_2$. It is of note that renal plasma flow, which increases for the first few hours after obstruction only starts to decline at about 4 hours after obstruction [Wright FS 1982] at a time when the leukocyte infiltrate is becoming evident (see Chapter 4). However, the possibility that platelets contribute...
to the enhanced thromboxane $A_2$ production following ureteral obstruction cannot be completely excluded by these studies.

The elimination of the leukocyte infiltrate from the obstructed kidney does not return the function of the post-obstructed kidney to normal. This is consistent with there being additional leukocyte-independent mechanisms operating in this model, such as the renin-angiotensin system [Yarger WE 1980, Ichikawa I 1985, Vaughan ED 1971, Kaloyanides GJ 1973]. Also it is of note that the elimination of the infiltrating macrophages by prior irradiation did not reduce the thromboxane $B_2$ excretion to baseline values. This is consistent with obstruction causing enhanced production of this vasoactive prostanoid by structures intrinsic to the kidney, such as the glomerular epithelium or mesangium: such a leukocyte independent source of thromboxane $A_2$ may also be capable of modulating renal haemodynamics.

Irradiation of the animals prior to obstruction also appears to influence the extent of the post-obstructive diuresis and natriuresis although the changes are small and the irradiated post-obstructed kidney still exhibits a significant diuresis and natriuresis compared to a non-obstructed kidney. Because of the greater glomerular filtration rate in the irradiated animals the filtered load of sodium was significantly higher in this group. Despite this, absolute sodium excretion remained unchanged, resulting in a lower fractional excretion of sodium. Similarly, there was decreased fractional water excretion in the irradiated animals. These parameters correlated with the urinary excretion rate of prostaglandin $E_2$. It is possible prostaglandin $E_2$ and other macrophage derived products could modulate transport processes in the renal epithelium [Kohan DE 1988]. However, following release of bilateral ureteral obstruction an osmotic diuresis secondary to urea accumulation contributes to the salt
and water loss [Maher JF 1963], and there may be elevated levels of circulating natriuretic factors [Parkerson ML 1989c]. Such extrarenal factors contributing to the diuresis would not be modified by prior elimination of the cellular infiltrate by irradiation and may explain why the effect of leukocyte depletion on tubular function was relatively minor.
CHAPTER 7

EVIDENCE FOR TWO DISTINCT SITES OF PROSTANOID PRODUCTION FOLLOWING BILATERAL URETERAL OBSTRUCTION.

7.1 Introduction.

The influx of leukocytes, predominantly macrophages, into the cortex and medulla of the kidney following bilateral ureteral obstruction is associated with an enhanced thromboxane B₂ excretion in the urine and the decline in glomerular filtration rate that occurs. These macrophages appear to play a functionally significant role in modulating post-obstructive glomerular haemodynamics in that their elimination, by prior irradiation of the animals, results in a significantly higher post-obstructed glomerular filtration rate and renal plasma flow and reduced thromboxane B₂ excretion (see Chapters 4, 5 and 6). However, it is of note that thromboxane B₂ excretion is not reduced back to control levels by prior irradiation, despite the total elimination of the infiltrate. In addition the marked enhanced production of prostaglandin E₂ that occurs following ureteral obstruction is only slightly reduced by prior irradiation of the animals.

This series of experiments demonstrates that there is an intrinsic renal source of enhanced prostanoid production following ureteral obstruction which is leukocyte independent. The relative functional contributions made by either infiltrating cells or intrinsic renal cells to the thromboxane A₂ induced alterations in renal haemodynamics following ureteral obstruction is also examined, using the selective thromboxane synthetase inhibitor OKY-046 [Hiraku S 1983].
CHAPTER 7 INTRARENAL PROSTANOID PRODUCTION

7.2 Methods.

7.2.1 Glomerular prostanoid production.

Two groups of animals were used. One group (n=12) underwent bilateral ureteral ligation as previously described, and the other (n=12) underwent a sham-operation in which the ureters were visualized, but not ligated. After 24 hours glomerular prostanoid production was measured as outlined in Chapter 3.

7.2.2 Clearance studies.

Adult male Lewis rats underwent bilateral ureteral obstruction and clearance studies 24 hours later as previously described in Chapter 3. Twenty-four hours prior to induction of bilateral ureteral obstruction (48 hours prior to clearance studies) one group of rats (n=7) received whole body irradiation of 1315 rads over 10 minutes (Gamma cell 40, Atomic Energy of Canada Ltd.). Eight rats received no irradiation but were studied in an otherwise identical fashion. As previously reported this irradiation protocol completely eliminates the leukocyte influx into both the cortex and medulla of the kidney following ureteral obstruction (see Chapter 6).

Two consecutive 20 minute clearance studies were performed as previously described and a sample of urine was collected on ice and stored at -70°C for subsequent thromboxane B₂ determination. The results of these two collection periods were averaged to give clearance results for the control period. Subsequently 5μg of the thromboxane synthetase inhibitor OKY-046 dissolved in 0.25 ml of normal saline, was administered via the tail vein catheter over 1 minute. This was followed by a sustained infusion OKY-046 at 40μg/hour; the drug was dissolved in the solution containing the inulin such that the overall infusion rate remained at 39μl/min. After 30 minutes, two subsequent timed clearance studies were performed and a further
sample of urine stored for subsequent thromboxane B2 analysis at -70°C.

7.3 Results.

7.3.1 Glomerular prostanoid production.

Glomeruli isolated from rats with 24 hours of bilateral ureteral obstruction exhibited enhanced production of both thromboxane B2 and prostaglandin E2 as compared to sham operated controls with the production rate of both prostanoids increasing by a factor of approximately 2 (see Table 7.1).

Table 7.1 Basal prostanoid production by isolated glomeruli from sham-operated control rats and rats with 24 hours of BUO.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>BUO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Thromboxane B2</strong></td>
<td>392.2</td>
<td>855.6</td>
</tr>
<tr>
<td>(pg/mg prot/hour)</td>
<td>±25.5</td>
<td>±31.1*</td>
</tr>
<tr>
<td><strong>Prostaglandin E2</strong></td>
<td>5242.1</td>
<td>10112.8</td>
</tr>
<tr>
<td>(pg/mg prot/hour)</td>
<td>±213.7</td>
<td>±697.1*</td>
</tr>
</tbody>
</table>

* P < 0.01 BUO vs CONTROL
7.3.2 Clearance studies.

There was no significant difference between the weights of the irradiated or non-irradiated rats, either prior to the onset of bilateral ureteral obstruction (241.4 ± 3.4 vs 236.3 ± 4.2 g) or following 24 hours of bilateral ureteral obstruction (236.3 ± 3.5 vs 228.4 ± 4.6 g), with the rats losing approximately 3% of their body weight following 24 hours of bilateral ureteral obstruction.

Prior irradiation of the animals so as to prevent the interstitial infiltrate resulted in a significantly lower excretion rate for thromboxane B₂, the stable metabolite of thromboxane A₂ (2.87 ± 0.56 vs 7.88 ± 2.14 pg/min). This confirmed the results reported in Chapter 6. This was associated with a significantly higher inulin clearance compared to the non-irradiated animals (2.78 ± 0.27 vs 1.49 ± 0.17 ml/min/kg BW). Administration of the thromboxane synthetase inhibitor OKY-046 to the animals which had been prior irradiated was able to produce a further decrease in thromboxane B₂ excretion from the obstructed kidney and also resulted in a further increase in the inulin clearance of that kidney (Figures 7.1 and 7.2).
Figure 7.1 Thromboxane B₂ excretion in rats following unilateral release of 24 hours of bilateral ureteral obstruction. Rats which had been prior irradiated had a significantly lower excretion rate of Thromboxane B₂ than non-irradiated rats (P < 0.05). Administration of the thromboxane synthetase inhibitor OKY-046 to the irradiated rats resulted in a further decrease in Thromboxane B₂ excretion (P < 0.005 pre OKY vs post OKY in irradiated animals).
Figure 7.2  Inulin clearance in rats following unilateral release of 24 hours of bilateral ureteral obstruction. Rats which had been prior irradiated had a significantly higher inulin clearance than non-irradiated rats (P < 0.001). Administration of the thromboxane synthetase inhibitor OKY-046 to the irradiated rats resulted in a further increase in inulin clearance (P < 0.005 pre OKY vs post OKY in irradiated animals).
CHAPTER 7 INTRARENAL PROSTANOID PRODUCTION

7.4 Discussion.

The obstructed kidney demonstrates enhanced arachidonate metabolism and generates increased levels of thromboxane A\textsubscript{2} [Morrison AR 1977, Morrison AR 1978]. As previously discussed the enhanced production of the vasoconstrictor prostanoid, thromboxane A\textsubscript{2} has been implicated in the pathogenesis of the decline in glomerular filtration rate following the onset of ureteral obstruction.

This thesis has provided evidence that infiltrating cells are a potent source of enhanced prostanoid (both thromboxane A\textsubscript{2} and prostaglandin E\textsubscript{2}) production following ureteral obstruction. The experiments described in this Chapter demonstrate that glomeruli are also a significant source of enhanced prostanoid production following ureteral obstruction. It has previously been reported that the production of thromboxane by glomeruli from rats with unilateral ureteral obstruction of 72 hours duration was greater from glomeruli obtained from the obstructed kidney than from glomeruli of the contralateral non-obstructed kidney [Folkert VM 1981]. The experiments reported here demonstrate that 24 hours of bilateral ureteral obstruction results in a highly significant increase in the production of both thromboxane A\textsubscript{2} and prostaglandin E\textsubscript{2} by glomeruli. The exact glomerular source of this enhanced prostanoid generation remains to be determined. It is unlikely to be glomerular leukocytes since these, in contrast to what is seen in the interstitium, are decreased in number following 24 hours of ureteral obstruction (as demonstrated in Chapter 4). Mesangial cells are known to contain cyclo-oxygenase activity [Harris RC 1990] and can generate thromboxane A\textsubscript{2} in response to a variety of stimuli [Zoja C 1990]. Thus, these cells could be the source of the increased production of prostanoids by isolated glomeruli from rats with obstruction.
CHAPTER 7 INTRARENAL PROSTANOID PRODUCTION

The intermediate steps underlying the mechanism of the increased synthesis of prostanooids by glomeruli in response to ureteral obstruction remain to be determined. There is an increased activity of both phospholipase A₂ and cyclooxygenase in animals with ureteral obstruction [Morrison R 1980, Needleman PA 1979]. Angiotensin II will increase the availability of arachidonic acid as a consequence of an effect of the hormone on the activity of phospholipase [Benabe JE 1982, Schlondorff D 1986] and may increase the activity of cyclooxygenase. Angiotensin II is released in response to ureteral obstruction [Vaughan ED Jr 1971a, Kaloyanides GJ 1973] and thus this may provide a mechanism for the enhanced production of thromboxane A₂ following ureteral obstruction. In support of this treatment of the animals with the angiotensin I converting enzyme inhibitor enalaprilat, prior to ureteral obstruction, effectively prevents the enhanced glomerular prostanooid production seen after ureteral obstruction [Yanagisawa H 1990a].

Confirmatory evidence of the pathophysiologic significance of thromboxane in ureteral obstruction has been provided by the demonstration that thromboxane synthetase inhibitors improve post-obstructive renal function, both by increasing renal plasma flow and K_f [Ichikawa 1985, Klotman PE 1986]. However, a beneficial effect of these compounds on post-obstructive renal function has not been found in all studies [Strand JC 1981, Loo MH 1987]. These differences may in part relate to the different animals and experimental conditions employed in the various studies. The effect of thromboxane synthetase inhibitors on post-obstructive glomerular filtration rate appears to be more pronounced when the compounds are given prior to the onset of the obstruction [Purkerson ML 1989a], but these agents also appear effective in improving renal function when administered following the release of the obstruction.
[Yarger WE 1980]. The source of the enhanced thromboxane generation within the kidney was previously unknown. Evidence from the chronically obstructed rabbit kidney suggested that infiltrating cells were important for the enhanced arachidonate metabolism to occur [Okegawa T 1983, Lefkowith JB 1984]. It is now clear that an infiltrate of leukocytes occurs in response to ureteral obstruction within as little as 4 hours following obstruction and that elimination of these cells both improves glomerular filtration rate and decreases thromboxane B\textsubscript{2} excretion, although not back to control levels (see Chapters 4-6).

The current study provides evidence for two distinct and functionally important sites for thromboxane A\textsubscript{2} generation from within the obstructed kidney. The fact that prior irradiation of the animals blunts the enhanced excretion of thromboxane B\textsubscript{2} following obstruction does indeed suggest that infiltrating leukocytes, which are predominantly macrophages, are a major source of thromboxane A\textsubscript{2} in this model. In addition it is clear that these infiltrating cells are functionally important since their elimination results in an improvement in post-obstructive renal function. However, the subsequent administration of the thromboxane synthetase inhibitor OKY-046 is able to further reduce thromboxane B\textsubscript{2} excretion. Since the increase in the interstitial cell number is totally prevented by prior irradiation this suggests that there must be also an intrinsic renal source of enhanced thromboxane A\textsubscript{2} generation, which does not depend on the presence of infiltrating cells. Furthermore this source of thromboxane A\textsubscript{2} is functionally significant since the administration of OKY-046 also resulted in a further increase in inulin clearance in the prior irradiated animals.

In summary the enhanced prostanoid generation that is seen following bilateral ureteral obstruction results from both an infiltration of the kidney by metabolically
active leukocytes and an enhanced production by glomeruli. Enhanced generation of the vasoconstrictor thromboxane \( A_2 \) by both of these sources appears to be functionally significant, as they both contribute to the decline in glomerular filtration rate that is seen following ureteral obstruction.
CHAPTER 8

RENAL CORTICAL RELEASE OF A SPECIFIC
MACROPHAGE CHEMOATTRACTANT IN RESPONSE TO
URETERAL OBSTRUCTION

8.1 Introduction.

Acute ureteral obstruction in rats results in the influx of mononuclear cells into both the cortex and medulla of the obstructed kidney. The infiltrate consists largely of macrophages, although small numbers of T cells are also present. The infiltration of the obstructed kidney by immune cells accounts in part for the alteration in renal function that occurs following ureteral obstruction, possibly via the production of vasoactive prostanoids such as thromboxane A₂.

The signal responsible for the recruitment of cells into the kidney following ureteral obstruction appears to be specific for macrophages since polymorphonuclear cells are not seen in the kidney following ureteral obstruction of less than 24 hours duration. The purpose of the studies presented here is to define the factor(s) responsible for the recruitment of cells into the obstructed kidney.

8.2 Methods.

8.2.1 Animal preparation.

Adult male Lewis rats underwent unilateral ureteral obstruction, under ether anaesthesia, as described in Chapter 3.

Initially, kidneys were harvested and analyzed for the production of a chemoattractant after four hours of ureteral obstruction, as this represents the time at which the mononuclear cell infiltrate first appears in the acutely obstructed kidney.
Subsequently kidneys were analyzed after 2, 12 and 24 hours of ureteral obstruction to determine whether the production of the chemoattractant varied with time. Chemotactic activity of kidney extracts for both neutrophils and macrophages was analyzed as described in Chapter 3.

8.2.2 Preparation of putative chemoattractant.

Initially the unmodified cell free supernatant was tested for chemotactic activity. Since chemotactic factors are in general proteins, small peptides, or lipids [Wilkinson P 1988], the supernatants were subsequently processed as outlined below, to elucidate the biochemical nature of the chemoattractant activity from the renal cortex of obstructed kidneys.

Effect of heating: To exclude the possibility that complement may be contributing to the macrophage migration, supernatant samples were heated to 56°C for 30 minutes prior to use. Additional samples were heated to 100°C for 5 minutes to denature protein in the supernatants, to further determine what effect this would have on the chemotactic activity. A precipitate that was formed during heating was removed by centrifugation before use. Three animals were studied in which the heated and non-heated samples of supernatant were run in parallel.

Extraction of supernatants: To further elucidate the nature of the chemoattractant activity that is produced by the obstructed kidney, supernatants from the cortices of obstructed or contralateral control kidneys were extracted over an octadecylsilane column (C18) (J.T. Baker Inc., Phillipsburg, NJ, USA). The octadecylsilane columns were conditioned prior to use by washing with 2 column volumes each of methanol (Fisher, St. Louis, MO, USA), deionized water, and 3% ethylenediamine-tetraacetic acid (Sigma Chemical, St. Louis, MO, USA) in water, followed by a final deionized
water rinse. The column primarily retains lipids, with large polypeptides eluting in the void volume and aqueous wash. Other compounds capable of hydrophobic interactions (e.g. some peptides) could also be retained. Obstructed kidneys were harvested 4 hours after obstruction unless otherwise stated. Supernatants were placed on the column at pH 7.4. (Reducing supernatant pH to 3-3.5 before extraction did not yield quantitatively more activity than pH 7.4.) The post-column effluent (aqueous fraction) was saved and the column washed with deionized water. Sequential elutions were then performed with petroleum ether, to extract non-polar lipids and free fatty acids, and methanol, to extract more polar lipids and other hydrophilic compounds retained by the column. HPLC grade organic solvents (Fisher, St. Louis, MO, USA) were used. The organic phases were dried in vacuo and resuspended to their original volume in RPMI with 0.25% albumin. Each fraction was then tested for chemotactic activity. Since both hydrophobic peptides and polar lipids would be eluted from the octadecylsilane column by the methanol, an additional extraction was performed on the methanol eluate to separate these two classes of compounds. A chloroform-aqueous partition was made using the method of Bligh and Dyer [Bligh EG 1959]. Each phase was dried in vacuo, resuspended in RPMI and tested for chemotactic activity.

To determine the relative contributions of chemotaxis (directed migration along a concentration gradient) and chemokinesis (accelerated random migration) to the total number of cells migrating, a checkerboard assay was also carried out [Wilkinson P 1986]. In this assay, migration of cells toward the putative chemoattractant was compared with migration occurring in the presence of a negative gradient (chemoattractant in the upper wells) or no gradient (chemoattractant in upper and
lower wells).

8.2.3 Effect of prior leukocyte depletion on kidney chemotactic activity.

As ureteral obstruction causes an influx of macrophages and macrophages themselves are a source of chemotactic substances, the effect of prior irradiation of the animals on the elaboration of the chemotactic substance was examined. Four animals received whole body irradiation 24 hours prior to unilateral ureteral obstruction as described in Chapter 4 (1300 rads over 10 minutes). This protocol is known to result in profound white cell depletion and to effectively prevent the leukocyte infiltration seen in response to ureteral obstruction (Chapter 6). Following ureteral obstruction cortices from the kidney were prepared as previously described and conditioned supernatants were extracted over an octadecylsilane column. The methanol eluates were tested for chemotactic activity and compared in parallel to the activity in the kidneys of two animals with ureteral obstruction which had not been irradiated.

8.3 Results.

The supernatants derived from short term culture of renal cortex from rat kidneys after 4 hours of obstruction demonstrated significantly greater chemoattractant activity for rat peritoneal macrophages than supernatants from contralateral, non-obstructed kidneys. The pooled data from 12 experimental animals are shown in Figure 8.1. When the supernatants of kidneys from two sham operated animals were assayed for chemotactic activity, this was found to be similar to that in the contralateral kidney of rats with unilateral ureteral obstruction (Figure 8.1).
Figure 8.1  Comparison of chemotactic activity from obstructed (OBS), contralateral control (CCK), and sham operated kidneys (SHAM). Kidneys were harvested after 4 hours of obstruction. Supernatants were used undiluted. Migration toward media alone is shown (RANDOM). Data is pooled from 12 rats except for SHAM where n=2. *P < 0.005, OBS vs CCK, OBS vs SHAM, P < 0.001, OBS vs RANDOM.
CHAPTER 8  MACROPHAGE CHEMOATTRACTANT

The supernatant from the cortex of the obstructed kidney attracted macrophages in a dose dependent manner (Figure 8.2). The migration of macrophages toward supernatants of cortices prepared from kidneys obtained 2, 4, and 24 hours after unilateral obstruction is shown in Figure 8.3. Chemoattractant production peaked early in the course of obstruction and declined with time.

When cortical supernatants from obstructed kidneys were tested in parallel with neutrophils (PMNs) and macrophages as responding cells, only macrophages exhibited a significant net migration (Table 8.1). Essentially no migration of PMNs occurred to the cortical supernatant. The PMNs did migrate (approximately 5 fold over random) toward a positive control of complement fragments in these experiments establishing the integrity of their locomotive capacity.

The effects of heating on chemotactic activity were assessed by running native supernatant in parallel with a sample of the same supernatant which had been heated to 56°C or 100°C. This was done in order to inactivate protein chemotactic factors. The chemoattractant activity of the supernatants was, however, not destroyed by heating (n=3). The results of a representative experiment are shown in Figure 8.4.
Figure 8.2  Dose-response curve of supernatant obtained from the cortex of an obstructed kidney. The supernatant was concentrated 2.8 fold (280%) by decreasing the volume of media the cortex was resuspended in such that there was 280 mg of cortex (wet weight) per ml of RPMI medium. Serial dilutions were then made. Net macrophage migration toward various dilutions of the supernatant is shown. Kidneys were obstructed for 4 hours prior to use. Measurements at each point were performed in triplicate. Random migration was 1267 ± 126 cells.
Figure 8.3 **Chemoattractant production as a function of duration of obstruction.** Macrophage migration toward supernatants obtained from kidneys obstructed for variable lengths of time is shown. All measurements were done in triplicate. Random migration was 1282 ± 82 cells.
### Table 8.1 Comparison of neutrophil (PMN) and macrophage (M\(\phi\)) migration toward the chemoattractant produced by the obstructed kidney.

<table>
<thead>
<tr>
<th></th>
<th>Total PMN migrated</th>
<th>Total M(\phi) migrated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media (random migration)</td>
<td>1285±335</td>
<td>1455±836</td>
</tr>
<tr>
<td>Cortical supernatant (CCK)</td>
<td>1040±26</td>
<td>2139±238</td>
</tr>
<tr>
<td>Cortical supernatant (OBS)</td>
<td>1017±52</td>
<td>3982±194*</td>
</tr>
</tbody>
</table>

* P < 0.05 OBS vs CCK cortical supernatants for macrophage migration.
There was no significant difference between migration towards media or CCK or OBS cortical supernatants for PMNs.
CCK, contralateral control kidney.
OBS, obstructed kidney.
Figure 8.4  Effect of heating on chemotactic activity of supernatants obtained from obstructed kidneys. OBS represents unheated supernatant from obstructed cortex. OBS 56°C was heated to 56°C for 30 minutes; OBS 100°C was heated to 100°C for 5 minutes. There are no significant differences among any of the conditions.
CHAPTER 8 MACROPHAGE CHEMOATTRACTANT

To further characterize the nature of the chemoattractant, supernatants were extracted on an octadecylsilane column. Figure 8.5 compares the chemotactic activity eluting in the post-column effluent (aqueous phase), the petroleum ether phase, and the methanol phase. Several points are illustrated in this figure. The peak activity stimulating macrophage migration eluted in the methanol fraction of supernatants from obstructed kidneys. This activity was significantly greater than the corresponding activity in the methanol fraction of supernatants from contralateral control kidneys. The aqueous and ether fractions had measurable activity, but there was no difference between obstructed and contralateral kidneys. Thus, the chemotactic activity specific to obstruction eluted with methanol. The methanol extract of supernatant from the obstructed renal cortex maintained a dose-response relationship for macrophage migration (Figure 8.6). The production of the methanol elutable chemotactic activity was again dependent on the duration of obstruction as illustrated in Figure 8.7, with peak biological activity being found between 4 and 12 hours.

Separating the methanol fraction into an organic and aqueous phase with chloroform and water resulted in chemotactic activity partitioning into each phase. The chloroform (i.e. lipid) phase contained a modestly larger fraction of the total activity (60 ± 5% vs 40 ± 5%, n=4, P < 0.05).

Investigation of the dependence of macrophage migration on the presence of a chemoattractant gradient was carried out to determine the relative contributions of chemotaxis and chemokinesis toward net cell movement. The results of this assay are shown in Figure 8.8. Reversing or abolishing the chemoattractant gradient prevented the migration of macrophages through the filter.

-144-
Figure 8.5  Comparison of the chemotactic activity of the fractions eluted from a C18 column. Supernatants extracted over the column were obtained from cortical homogenates of kidney obstructed for 4 hours (OBS) or from the contralateral control kidney (CCK). RANDOM represents migration toward media processed in the same fashion as supernatants. The AQUEOUS fraction represents the post-column supernatant; the ETHER and METHANOL fractions represent subsequent column elutions with petroleum ether and methanol, respectively. Extracts were reconstituted in media to be the equivalent concentration as undiluted cortical supernatants. Data are pooled from 3-5 animals. *P < 0.04, OBS methanol vs CCK methanol, P < 0.005, OBS methanol vs RANDOM. Other comparisons are not significant.
Figure 8.6  Dose-response curve of the methanol eluate from supernatant of obstructed renal cortex. Supernatants were passed over a C18 column as detailed in Methods. The material eluted from the column with methanol was concentrated two fold (200%) and serial dilutions then made. Macrophage migration toward each dilution was assessed. Kidneys were analyzed after 4 hours of obstruction. Measurements were made in triplicate.
Figure 8.7 Chemoattractant production as a function of duration of obstruction in methanol extracts of cortical supernatants obtained from obstructed kidneys. Kidneys were obstructed for variable lengths of time. Net macrophage migration was assessed for each time point.
Figure 8.8  **Checkerboard assay to assess chemotaxis vs. chemokinesis.** Total macrophage migration was measured. Conditions were: random migration toward media alone (BLANK), migration toward the methanol extract from obstructed cortical supernatant (METHANOL EXTRACT), migration in the presence of equal concentrations of obstructed methanol extract in the upper and lower wells of the chemotaxis chamber (NO GRADIENT), and migration with methanol extract in the upper wells and media alone in the bottom wells (GRADIENT REVERSED). Each condition was measured in triplicate.
In order to assess the relative contributions of intrinsic renal cells and infiltrating leukocytes to the chemotactic activity found in the conditioned supernatants of obstructed cortical cultures, depletion of leukocytes by irradiation prior to ureteral obstruction was performed. The methanol fractions derived from obstructed kidneys of irradiated animals resulted in an average macrophage migration of 3142 cells. Supernatants derived from kidneys of non-irradiated obstructed rats run in parallel yielded an average macrophage migration of 5162 cells. Random migration in these experiments was 1700 cells. Thus eliminating the white cell infiltrate resulted in an ≈ 40% decrease in the chemotactic activity of cultured cortical supernatants.

8.5 Discussion.

The acutely obstructed rat kidney generates a chemoattractant for rat macrophages. The production of this factor by the obstructed kidney is significantly enhanced over that of the contralateral control kidney. There was no enhanced production of this factor by the kidneys of sham operated animals. This activity is truly chemotactic and not just chemokinetic, in that macrophage migration is dependent upon the presence of a chemoattractant gradient.

The chemoattractant(s) is specific for macrophages, as there was no induction of neutrophil migration by either the native or methanol extracted supernatants. This correlates well with the observation that neutrophils do not appear in the acutely obstructed rat kidney.

The production of the chemotactic factor occurs early in the course of ureteral obstruction (peaking between 4 and 12 hours) and appears to decline with time.
is consistent with the observations on the kinetics of arrival of the infiltrating cells following obstruction. Following ureteral obstruction the infiltrate starts around 4 hours and plateaus between 12 and 24 hours, indicating that the signal to recruit these cells into the kidney declines with prolonged ureteral obstruction. The experiments following leukocyte depletion indicate that some (≈ 40%) of the chemotactic activity found in the cortical supernatants could be accounted for by chemotactic substances released from the infiltrating cells themselves. This would provide a positive feedback mechanism whereby the signals responsible for the early recruitment of cells following ureteral obstruction are amplified. This appears to be a self limiting response since the recruitment of cells does plateau. However the majority of the chemotactic signal (≈ 60%) appears to originate from intrinsic renal cells.

The chemotactic factor isolated in the current study is heat stable to boiling which suggests that it is not complement or a protein with a tertiary structure necessary for activity. The fractionation of supernatants from obstructed cortex on the C18 column provides additional insight into the chemical nature of the chemoattractant. Although measurable activity was found in the aqueous and petroleum ether fractions, this activity does not appear to be specific for obstruction as there was no difference between samples from the obstructed or contralateral kidney. It is likely that this activity represents non-specific effects of tissue isolation, trauma, and ischemia. The lack of specific activity in these fractions implies that the chemoattractant is not a protein, phospholipid, fatty acid, or non-polar lipid [Powell W 1980]. Chemotactic activity specific for obstruction was found in the methanol fraction. Methanol will elute moderately polar lipids and other hydrophilic compounds from the column [Powell W 1980]. The further fractionation of the
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methanol eluate into an aqueous phase and chloroform phase, both of which have biological activity, suggests that either two different chemoattractants are produced, or that the lipid extraction was partial. The factor partitioning into the chloroform is lipid in nature. Prior studies in this thesis and of others have demonstrated enhanced production of cyclo-oxygenase products (thromboxane A₂ and prostaglandin E₂) from the obstructed kidney [Okegawa G 1983]. However, neither eicosanoid has chemotactic activity for monocytes [Campbell PB 1988, McClatchey W 1976]. In addition, acute alterations in the lipid composition and turnover of the renal cortex have been demonstrated following ureteral obstruction [Tannenbaum J 1983, Morrissey J 1986]. It is thus conceivable that perturbations in lipid metabolism during obstruction are associated with the release of a lipid chemoattractant. The release of this lipid chemoattractant may represent a novel pathway of the renal response to stress, in which monocytes are induced to migrate into the kidney in the absence of inflammatory mediators of injury, such as complement.

The factors causing an obstructed kidney to release a chemoattractant for macrophages remains unknown, but it is possible that an increase in tubular pressure or membrane stretch in response to ureteral obstruction cause the cells to release a chemoattractant. It is of note that the infiltrating cells in obstruction localize predominantly around distal tubular structures, which would be the cells subjected to the most mechanical stress following ureteral obstruction.

In summary, this experiment provides evidence that acute ureteral obstruction results in the release of a specific monocyte chemoattractant(s) from the affected kidney. Initial analysis suggests that at least one of the factors released is lipid in nature. These studies are significant in establishing the relationship between the
immune system and renal lesions not previously considered to be immunologic in nature. It suggests the existence of a signalling mechanism whereby macrophages can be induced to migrate into the renal interstitium in response to physiological stress or injury. This may be particularly significant in light of the apparent ability of infiltrating interstitial leukocytes to modulate glomerular filtration and renal blood flow.
CHAPTER 9

OBSTRUCTIVE NEPHROPATHY: FROM MECHANICAL DISTURBANCE TO IMMUNE ACTIVATION.

9.1 Summary

The alterations in renal function that occur as a result of simple mechanical obstruction to the renal tract are complex and are not merely a consequence of impairment to urine flow. The effects of urinary tract obstruction are a result of variety of factors with complex interactions, resulting in alterations in both glomerular haemodynamics and tubular function, with alterations in monovalent and divalent ion handling.

More than ten years ago, chronic urinary tract obstruction in rabbits was found to be associated with a proliferation of interstitial fibroblasts and an infiltration of mononuclear cells [Nagle RB 1973, Nagle RB 1976]. More recently these changes have been linked to the increase in prostaglandin E\textsubscript{2} production by the chronically hydronephrotic rabbit kidney [Davis BB 1983]. It has been postulated that the infiltration of the renal parenchyma by mononuclear cells may affect the augmented release of thromboxane A\textsubscript{2} and prostaglandin E\textsubscript{2} in response to bradykinin [Okegawa T 1983] or endotoxin [Lefkowith JB 1984].

The interstitial leukocyte infiltrate: In this thesis a rat model of acute and reversible ureteral obstruction was used to define the kinetics of the cellular infiltrate that is seen following ureteral obstruction. In addition the cell type making up the infiltrate was determined. In this model there is no necrosis and renal functional abnormalities are transient. The leukocyte influx appears to be one of the earliest responses of the kidney to ureteral obstruction, occurring within four hours of...
obstruction, with the peak response occurring at 12 hours after which a plateau is observed. The leukocytes form distinctive rings around the tubules, particularly distal tubules. Of note, the infiltrate is observed not only in the cortex, but also in the medulla which is normally almost completely devoid of resident leukocytes, presumably due to its hypertonic environment. The mononuclear cell infiltrate observed in obstruction consists predominantly of macrophages. The second major leukocyte population consists of T-lymphocytes of the cytotoxic, suppressor cell subclass. Some degree of selectivity is implied by the fact that T-lymphocytes of the helper type do not constitute a significant portion of the infiltrate despite the fact that they predominate in the peripheral circulation [Mampaso F 1983]. B-lymphocytes do not appear in the renal interstitium. Neutrophils are also absent from this infiltrate, consistent with this acute lesion not being associated with tissue necrosis.

**Haemodynamic changes:** The kinetics of the macrophage and leukocyte invasion temporally correlates with both the decline in glomerular filtration rate and the enhanced thromboxane production by the kidney seen in response to ureteral obstruction. Since leukocytes are a potent source of thromboxane A₂ and possibly other vasoactive compounds these phenomena could be causally related. In support of this total body irradiation of rats prior to the onset of obstruction so as to prevent the leukocyte infiltrate in the kidney, both reduces thromboxane B₂ excretion in the urine and significantly improves renal haemodynamics after release of 24 hours of obstruction. By contrast, irradiation had no effect on renal morphology or function in normal rats. This implies that infiltrating leukocytes contribute to the decline in glomerular filtration rate and renal plasma flow seen after obstruction, possibly via the production of vasoactive prostanoids such as thromboxane A₂.
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The elimination of the leukocyte infiltrate from the obstructed kidney, however, does not return the function of the post-obstructed kidney to normal. This is consistent with there being additional, leukocyte-independent, mechanisms operating in this model. Also, it is of note that elimination of the infiltrating macrophages by prior irradiation did not completely reduce the thromboxane B₂ excretion in the urine to baseline levels. This is consistent with obstruction causing enhanced production of these vasoactive prostanoids by structures intrinsic to the kidney, such as glomerular epithelium or mesangium. Glomeruli isolated from rats with ureteral obstruction demonstrated an enhanced ability to produce a prostanoids, including thromboxane A₂, compared to glomeruli from non-obstructed kidneys. Such leukocyte independent sources of thromboxane A₂ also appear to be capable of modulating renal haemodynamics.

Changes in tubular function: As previously discussed, abnormalities in tubular function are common in urinary tract obstruction [Yarger WE 1982], and results in changes in renal handling of a variety of electrolytes as well as alterations in the regulatory control of water excretion. There is a decreased ability to concentrate the urine, and the reabsorption of sodium and other solutes such as phosphorus, magnesium and calcium ions is altered, as is the secretion of hydrogen and potassium. The mechanism for these alterations in function which result in the syndrome commonly referred to as "post-obstructive diuresis", remains unclear, but most of the evidence points to the major site of damage being in the distal nephron. With protracted periods of obstruction there are morphological changes in the tubules and the resultant loss of function, thus, is not surprising. However, even with short periods of obstruction, when there is minimal histological change, the alterations in
CHAPTER 9 OBSTRUCTIVE NEPHROPATHY

Tubular function are marked. This would suggest that following obstruction various modulators of tubular epithelial function are generated and act within the kidney. Since ureteral obstruction is associated with an interstitial infiltrate which predominantly rings distal tubular structures it is possible that leukocyte derived mediators may be contributing to the post-obstructive alterations in renal tubular function. It is of note that in more classical forms of interstitial nephritis sodium and water wasting, phosphate wasting and impaired hydrogen ion secretion are all well recognized features [Cogan MC 1982, Vaziri ND 1979]. In support of this irradiation of the animals so as to prevent the interstitial infiltrate does produce an amelioration in the post-obstructive tubular dysfunction, although these changes are relatively minor and it is apparent that other mechanisms are likely to play a role. It is of note however that a correlation was observed between the excretion of prostaglandin E₂ and the extent of the post-obstructive diuresis suggesting a possible causal link between these parameters.

**Reversibility:** The infiltration of the kidney by leukocytes is a slowly reversible process requiring several days to revert to near normal levels once the obstruction has been released. If the obstruction is not relieved, however, the infiltrate of macrophages, with their known potential to induce fibroblast proliferation and scar formation persists. It is likely that this immunological infiltrate may have long-term adverse consequences on renal function and structure. Indeed, it has been shown that in patients with ureteropelvic obstruction focal segmental glomerulosclerosis is a common histological finding in the obstructed kidney [Steinhardt GF 1988b]. This focal segmental glomerulosclerosis is found in areas closely associated with intense interstitial and peri-glomerular inflammation.
Production of a macrophage specific chemoattractant: The demonstration that the cortex of the obstructed kidney elaborates a unique chemoattractant that is specific for macrophages provides a mechanism for the recruitment of these biologically active cells into the kidney following ureteral obstruction. This substance behaves biochemically as a lipid, but has not yet been fully characterised. In addition it remains possible that there are other chemotactic signals released by the obstructed kidney which may also be responsible for recruiting macrophages into the obstructed kidney. Such signals remain to be fully defined. How obstruction to the urinary tract results in the production and or release of chemotactic substances by the kidney is also unclear, but it would seem likely that the stimulus is either an increase in pressure or cell membrane stretch as the tubules dilate following obstruction.

Glomerular changes: The pattern of change in glomerular macrophage number following ureteral obstruction is somewhat different. There is a relative depletion of resident leukocytes from the glomeruli associated with the leukocyte invasion of the interstitium. The destination of the glomerular leukocytes, which are resident mesangial macrophages [Schreiner GF 1981], is unclear, but one possibility is that they are induced to migrate from the glomerulus to the interstitium via the mesangial stalk in response to signals from the obstructed interstitium. After release of the obstruction, the glomerulus is repopulated with resident macrophages which accumulate to a level significantly increased when compared to control glomeruli. The increase is comparable to the degree of macrophage infiltration observed in a model of moderate glomerulonephritis induced by nephrotoxic serum [Schreiner GF 1984].

The exact pathophysiologic significance of the changes in glomerular
macrophage number following ureteral obstruction remains to be determined. In the rat, following release of short term (less than 30 hours) of ureteral obstruction, recovery of glomerular filtration rate is complete 7-9 days later [McDougal WS 1972]. Although this would suggest that short term obstruction is completely reversible and that most of the early alterations in renal function are functional, there is indirect evidence to suggest that recovery may not be a consequence of a homogeneous recovery in the single nephron glomerular filtration rate (SNGFR) of all nephrons. Indeed, when SNGFR and the number of filtering nephrons were determined using a modification of Hansen's technique, a decrease in the total number of filtering nephrons was found in the post-obstructed kidney, such that only 85% of the nephrons in the post-obstructed kidney were filtering, compared to 100% in the contralateral kidney. Thus, the normalization of whole kidney glomerular filtration rate occurs at the expense of hyperfiltration (increase in SNGFR) in the remaining functional nephrons, and there is a permanent decrement in the total number of functional nephrons [Bander SJ 1985]. It is interesting to speculate that this permanent loss of nephrons following release of only short periods of obstruction may be the result of the macrophage infiltration of the glomeruli seen following the release of the obstruction.

Thus work presented in this thesis demonstrates that a leukocyte infiltrate plays a key role in the alterations in renal function that occur as a result of ureteral obstruction, suggesting that ureteral obstruction should now be considered to have an immunological component.
CHAPTER 9 OBSTRUCTIVE NEPHROPATHY

RECENT INSIGHTS INTO THE PATHOPHYSIOLOGY OF URETERAL OBSTRUCTION.

The work presented in this thesis was carried out between 1986 and 1989. The observations made in this thesis on the pathophysiologic significance of infiltrating cells in ureteral obstruction have been subsequently confirmed and extended by both the group with which I worked and others. The purpose of the following sections is to review the more recent changes in the understanding of the pathophysiology of ureteral obstruction and in particular review the further work that has been performed on the role of infiltrating cells in both the acute and chronic changes that follow on from obstruction to the urinary tract.

9.2 Vasoactive compounds. New insights into their role in the haemodynamic changes following ureteral obstruction.

The perturbations in renal haemodynamics have been attributed to alterations in both vasodilator and vasoconstrictor compounds. These principally include modulation of prostamoid production including vasodilator and vasoconstrictor (predominantly thromboxane A₂) prostaglandins and alterations in the renin-angiotensin axis as discussed in Chapter 1. More recent studies using newer and more specific pharmacological blocking agents have confirmed a central role for these compounds in post-obstructive renal functional changes.

Thromboxane A₂. Following release of 24 hours of unilateral ureteral obstruction rats which had been pre-treated with and subsequently maintained on the thromboxane A₂ receptor antagonist GR32191 (a Glaxo compound) had significantly greater insulin
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and PAH clearances with a reduction in renal vascular resistance compared to saline treated controls. In addition the histopathologic findings in the GR32191 treated rats were much less severe [Rinder CA 1994].

Renin-angiotensin axis. Following 24 hours of unilateral ureteral obstruction rats pretreated with the angiotensin converting enzyme Lisinopril or the angiotensin I receptor antagonist Losartan, had improved renal haemodynamics, lower mean arterial pressure, and normalized eicosanoid excretion rates compared to untreated controls. Renin expression was found in mesangial cells, along greater lengths of afferent arterioles and in dilated distal tubules and loops of Henle of the post obstructive kidney. In situ hybridization revealed that =20% more glomeruli in obstructed kidney over expressed renin mRNA than in the control kidney. Blood vessels of the obstructed kidney consistently showed greater expression of the genes coding for components of the renin angiotensin system than in control kidney, suggesting a role for local Angiotensin II production in the severe haemodynamic changes following ureteral obstruction [Pimentel JL Jr 1993].

Interactions between the renin-angiotensin axis and prostanoid production. Both the prostaglandin and renin-angiotensin axis are functionally linked. The increased release of renin is virtually abolished by pretreatment with the cyclo-oxygenase inhibitors indomethacin or meclofenamate [Blackshear JL 1978, Cadnapaphornchai P 1978], suggesting that the formation of vasodilatory prostaglandins such as prostacyclin or prostaglandin E₂ represents a necessary step for the release of renin from juxtaglomerular cells [Gerber JG 1981].

In addition the generation of eicosanoids is dependant on an intact renin-angiotensin system. As shown in Chapter 7, basal prostanoid production is greater.
in isolated glomeruli from rats with bilateral ureteral obstruction than in controls. Administration of an ACE inhibitor to rats with bilateral ureteral obstruction returns prostanoid production by isolated glomeruli to levels seen in glomeruli of control rats. Glomerular eicosanoid production in response to addition of exogenous angiotensin II or AVP in vitro is blunted in glomeruli from rats with ureteral obstruction, but this response is also restored to 'normal' after blockade by prior treatment of the rats with ACE inhibitors [Yanagisawa H 1990a]. These results indicate that endogenous angiotensin II has an important role in the increased synthesis of eicosanoids found in isolated glomeruli of rats with bilateral ureteral obstruction. Similar results have also been demonstrated in glomeruli from rats with unilateral ureteral obstruction [Yanagisawa H 1990b]. However, glomeruli isolated from rats with unilateral obstruction tend to produce more thromboxane B₂ than those from rats with bilateral obstruction; this may contribute to the more exaggerated vasoconstrictor response seen in unilateral compared to bilateral ureteral obstruction [Fukuzaki A 1993].

The changes in cell biology underlying the enhanced glomerular eicosanoid production following ureteral obstruction have recently been elucidated. Glomeruli isolated from rats with 24 hours of bilateral ureteral obstruction have a significantly increased activity of cyclo-oxygenase as a result of de novo synthesis of this enzyme. In addition there is a markedly augmented activity of prostaglandin E₂ isomerase and both prostaglandin I₂ and thromboxane synthases relative to glomeruli isolated from control rats. Similarly, the activity of membrane-bound 5-lipoxygenase, the active location of this enzyme, is significantly greater in glomeruli isolated from rats with ureteral obstruction. Thus, ureteral obstruction enhances the glomerular production of eicosanoids via the activation of enzymes in both the cyclo-oxygenase and
5-lipoxygenase pathways [Yanagisawa H 1993]. The mechanisms responsible for enhanced production of vasodilator prostanoids by glomeruli from rats with bilateral ureteral obstruction is an increase in the activity in glomerular membranes of L-alpha-Phosphatidylcholine specific and L-alpha-phosphatidylethanolamine specific phospholipase A2 as well as an increase in both the activity and amount of cyclooxygenase. Treatment of the animals prior to ureteral obstruction with either an ACE inhibitor or a thromboxane synthase inhibitor prevents this increase in both cyclooxygenase and the L-alpha-phosphatidylethanolamine specific phospholipase A2. In contrast L-alpha-Phosphatidylcholine specific phospholipase A2 activity is further increased in glomerular membranes from rats pretreated with the two drugs. Thus increased synthesis of vasodilatory eicosanoids by glomeruli from rats with ureteral obstruction may be mediated by enhanced activities of L-alpha-phosphatidylethanolamine specific phospholipase A2 and cyclooxygenase. These are apparently stimulated by the vasoconstrictors angiotensin and thromboxane [Yanagisawa H 1991]. Interestingly dietary protein intake affects the increases in glomerular eicosanoid production by glomeruli from rats with ureteral obstruction. High protein diets augment glomerular prostanoid production apparently by altering the activity of the cyclo-oxygenase pathway which in turn mainly dependant on the activity of the renin-angiotensin system. Thus, protein content in a diet may modify an alteration in renal haemodynamics caused by ureteral obstruction by changing the glomerular production of eicosanoids and the activity of the renin-angiotensin system [Yanagisawa H 1994]. This observation may in part explain the beneficial effect of low protein diets on the haemodynamic alterations following ureteral obstruction [Ichikawa I 1985].
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OTHER VASOACTIVE COMPOUNDS IN URETERAL OBSTRUCTION.

ATRIAL NATRIURETIC PEPTIDE: Saline loading so as to effect volume expansion following
release of ureteral obstruction results in an improvement in post-obstructive renal
function, an effect which can be reproduced by the administration of exogenous atrial
peptide [Harris KPG 1991]. It has been suggested that some of the differences
observed between the response to bilateral and unilateral ureteral obstruction may be
explained by differences in atrial peptide levels. Plasma atrial peptide levels are
higher following bilateral than unilateral ureteral obstruction. Administration of atrial
peptide causes a fall in renal vascular resistance and induces a dramatic increase in
the production of the stable metabolite of the vasodilator prostacyclin
(6-ketoprostaglandin F1α) without affecting production of thromboxane B2.
Indomethacin blunts but does not prevent the response to atrial peptide suggesting that
atrial peptide may act as a potent renal vasodilator following ureteral obstruction by
both a direct action and secondarily through stimulation of renal prostacyclin
production [Himmelstein SI 1990].

VASOPRESSIN: Rats with bilateral (but not unilateral) ureteral obstruction of 24 hours
duration have elevated plasma levels of ADH compared to control animals:
pretreatment with a specific antagonist of the V1-type receptor for ADH results in an
improvement in both the glomerular filtration rate and the effective renal plasma flow
following release of ureteral obstruction and significantly lowers mean arterial
pressure suggesting that high levels of ADH may play a role in the decrease in the
glomerular filtration rate and effective renal plasma flow in this condition [Reyes AA
1991a].

PLATELET-ACTIVATING FACTOR: This is a powerful vasodilator with important effects on

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kidney function which may be expected to protect the kidney against the actions of vasoconstrictor compounds. However, following 24 hours of bilateral ureteral obstruction infusion of PAF directly into the renal artery decreases the glomerular filtration rate and effective renal plasma flow; this effect may result from PAF inducing further intrarenal production of thromboxane A₂, as when the production of thromboxane A₂ has been inhibited, PAF administration increases renal function as a result of its unopposed vasodilatory properties. Pretreatment of animals with a PAF antagonist worsens post-obstructive renal function further supporting a role for endogenous PAF production as an intrarenal vasodilator in obstructive nephropathy [Reyes AA 1991b].

**Nitric Oxide:** Intrarenal generation of the vasodilator nitric oxide may protect against the fall in renal function following ureteral obstruction. The administration of N(w)NAME, an inhibitor of nitric oxide synthesis, after unilateral release of 24 hours of bilateral ureteral obstruction decreases renal function and rats given N(w)NAME prior to obstruction have no measurable renal function 24 hours latter [Reyes AA 1992]. Similar results are also seen following unilateral ureteral obstruction [Chevalier RL 1992]. In bilateral ureteral obstruction (but not unilateral ureteral obstruction) the availability of the substrate for nitric oxide synthesis (L-Arginine) is rate limiting since administration of L-Arginine either before or immediately after unilateral release of 24 hours of bilateral ureteral obstruction significantly improves renal function [Reyes AA 1992a]. Plasma levels of L-Arginine have been found to be low following bilateral ureteral obstruction [Reyes AA 1994a].

**Endothelin:** The administration of a specific anti-endothelin antibody to rats with bilateral ureteral obstruction significantly improves post-obstructive renal function
In the setting of bilateral ureteral obstruction the net role of the renal artery endothelium is vasodilatory i.e. the activity of nitric oxide exceeds that of endothelin, since mechanical denudation of the main renal artery endothelium results in worse renal function [Reyes AA 1992b]. In contrast, in normal rats denudation of the main renal artery endothelium has no effect on renal function.

**Leukotrienes:** Leukotrienes are products of the 5-lipoxygenase pathway of arachidonic acid metabolism that possess potent inflammatory properties. Isolated glomeruli from rats with bilateral ureteral obstruction produce significantly greater amounts of leukotriene B₄ than glomeruli from sham-operated rats. Rats given MK886, an inhibitor of the 5-lipoxygenase enzyme prior to ureteral obstruction have significantly better renal function following the release of the ureteral obstruction 24 hours later, suggesting that increased synthesis of leukotrienes also plays a role in the haemodynamic changes seen after ureteral obstruction [Reyes 1992c].

### 9.3 Infiltrating cells following ureteral obstruction. New insights into mechanisms of recruitment

Work in this thesis clearly demonstrated that the obstructed kidney generates a lipid chemoattractant which is macrophage specific. In addition there is now an increasing body of work supporting the concept that a lipid like chemoattractant plays a pivotal role in recruiting macrophages into the kidney in various renal diseases; for example a lipid chemoattractant is produced by tubular cells in response to proteinuria [Kees Folts D 1994]. To date however the exact biochemical nature of this substance has not been determined.
The ability to recruit macrophages into sites of tissue injury is likely to be a process of such fundamental importance that there may be many potential mechanisms to bring it about. Certainly several other chemoattractants have been demonstrated to be produced by the kidney and to possibly play a role in macrophage recruitment in other renal diseases. These include a number of protein chemoattractants or chemokines. Using a model of unilateral ureteral obstruction Diamond and co-workers have confirmed the observations made in this thesis that there is a large time dependant increase in interstitial macrophage number following ureteral obstruction. Messenger RNA for the chemokine monocyte chemoattractant peptide-1 (MCP-1) could be detected in the obstructed kidney from 12 to 96 hours following the onset of ureteral obstruction but was absent in the contralateral control kidney. In addition MCP-1 protein could be detected on the apical tubular membrane using immunohistochemical techniques but was absent in the control kidney [Diamond JR 1994]. The exact significance of apical tubular membrane expression of MCP-1 to recruitment of macrophages into the interstitium of the kidney remains to be determined however.

Recently evidence has been provided for a role for osteopontin, a protein with potential chemoattractant activity, in the accumulation of macrophages in the kidney following ureteral obstruction. Increased osteopontin mRNA levels are found in obstructed kidneys as early as 4 hours after unilateral ureteral obstruction and increase further after 12, 24, 48, and 96 hours. These increments in renal cortical osteopontin mRNA are paralleled by the increase in the number of cortical renal interstitial macrophages. X-irradiation significantly diminishes the macrophage infiltrate but does not alter renal cortical osteopontin mRNA expression. Using immunohistochemical
techniques, focal labelling of osteopontin can be detected in both tubular and
Bowman's capsular epithelium in obstructed kidneys as early as 4 hours after
obstruction and by 96 hours this is more intense and diffuse. These observations
would support the contention that osteopontin is involved in the accumulation of
macrophages within the peri-tubular and peri-glomerular interstitium in the obstructed
renal cortex [Diamond JR 1995a].

Although not all mechanisms by which macrophages enter the renal
interstitium have been elucidated inhibition of monocyte/macrophage function provides
a potential therapeutic target for the treatment of renal dysfunction following ureteral
obstruction. Recent work has demonstrated that it may be possible to modify the
extent of the macrophage infiltrate following ureteral obstruction in several different
ways.

The cortical leukocyte infiltrate following ureteral obstruction is associated
with higher levels of malondialdehyde, an index of lipid peroxidation and decreased
levels of reduced glutathione and increased levels of oxidized glutathione.
Administration of the lipid lowering agent and anti-oxidant Probucol for two weeks
prior to ureteral obstruction results in significantly higher levels of reduced glutathione
in the obstructed kidneys, a reduction in the severity of the leukocyte infiltrate and
greater inulin and PAH clearances at three to five hours and three days following
release of bilateral ureteral obstruction compared to non treated rats. Reduction in
cholesterol using Lovastatin (a cholesterol lowering agent without anti-oxidant
properties) does not produce a similar beneficial effect on renal function, suggesting
the effect seen with probucol may be due to ability of this drug to prevent the
accumulation of reactive oxygen metabolites and/or decrease the number of leukocytes.
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infiltrating the renal cortex [Modi KS 1990].

Administration of 1% L-arginine in the drinking water decreases the infiltration by macrophages into the kidneys of rats in response to bilateral ureteral obstruction. This is associated with an improvement in post-obstructive renal function. The random migration of peritoneal macrophages obtained from rats with obstruction given L-arginine prior to obstruction is significantly lower than that of peritoneal macrophages obtained from similar rats given tap water alone prior to obstruction. This would suggest that L-arginine may cause a decrease in macrophage infiltration by directly inhibiting macrophage chemotaxis, a hypothesis supported by the observation that L-arginine will inhibit macrophage chemotaxis in response to activated serum in vitro [Reyes AA 1994b].

The macrophage infiltration of the kidney following ureteral obstruction also appears to be attenuated by the administration of the angiotensin converting enzyme inhibitor enalapril [Ishidoya S 1995], a manoeuvre which is known to improve post-obstructive renal function in this model [Purkerson 1989a]. Interestingly however, treatment with an angiotensin II receptor antagonist, whilst still improving post-obstructive renal function, does not prevent macrophage infiltration to the same degree [Ishidoya S 1995].

9.4 Infiltrating cells following ureteral obstruction. New insights into pathophysiologic significance.

This thesis provides evidence that an interstitial infiltrate of macrophages is responsible in part for the acute alterations in renal function that are seen following short term (< 24 hours) ureteral obstruction. Others have now confirmed that a
macrophage infiltrate is a prominent feature of ureteral obstruction [Diamond JR 1994 and 1995] and that manoeuvres which bring about a reduction in the infiltrate are associated with an improvement in post-obstructive renal function [Modi KS 1990, Reyes AA 1994b, Ishidoya S 1995].

In addition to modulating acute changes in renal function the presence of large numbers of biologically active macrophages within the kidney could also result in chronic damage and hence permanent loss of renal function. It is clear from studies in experimental animals that after prolonged ureteral obstruction permanent atrophy of the kidney can result which can not be influenced by the relief of the obstruction [Gonnermann D 1990]. Such changes would be of relevance to the chronic loss in renal function which often accompanies chronic obstruction to the urinary tract in humans [Better OS 1973]. Infiltrating macrophages are now thought to play a central role in the development of glomerulosclerosis and interstitial fibrosis in a variety of different experimental and human renal diseases [Van Goor H 1994]. Macrophages are a potent source of growth factors and cytokines many of which have the potential to play a role in the development and progression of fibrotic and sclerotic changes that occur in the chronically obstructed kidney. For example Interleukin-1 derived from macrophages can both increase mesangial cell proliferation [Lovett DH 1983] and stimulate the synthesis of type IV collagen by glomerular epithelial cells [Torbohm I 1989]. Macrophage derived products have also been shown to increase the production of extracellular matrix proteins by mesangial cells in culture [Harris KPG 1995]. In addition macrophages are capable of releasing a variety of other products which could potentially injure the glomerulus, including proteolytic enzymes (collagenase, elastase), reactive oxygen species, cyclo-oxygenase and lipoxygenase.
products, platelet derived growth factor, coagulation factors and platelet activating factors [Nathan CF 1987].

Chronic unilateral ureteral obstruction (3-5 days) results in interstitial fibrosis of the affected kidney with increased expression for collagen type IV in both the tubular basement membrane and the interstitial space. Staining for collagen types I and III is also increased in the interstitial space of the obstructed kidney, although glomerular staining for these matrix protein is unaltered. Treatment of rats with angiotensin converting enzyme inhibitors ameliorates the increased production of extracellular matrix proteins in the tubulointerstitium following ureteral obstruction [Kaneto H 1994]. As previously discussed angiotensin converting enzyme inhibition reduces the macrophage infiltrate following ureteral obstruction and their beneficial effects on interstitial fibrosis may result from the reduction in the number monocytes/macrophages capable of secreting the profibrotic cytokines.

While several cytokines may initiate fibrogenesis, TGFβ is considered to be one of the prime profibrogenic cytokines central to the development of fibrosis in many organs including the kidney [Border WA 1994]. TGFβ mRNA levels are increased significantly in the obstructed kidney after three days of unilateral ureteral obstruction compared to control (unoperated rats) kidneys and this increase is found in tubular cells rather than glomeruli. Inhibition of thromboxane synthetase does not affect the changes in TGFβ mRNA in the obstructed kidney but inhibition of the angiotensin converting enzyme significantly blunts (although does not completely abrogate) the increase [Kaneto H 1993]. These observations would suggest that following ureteral obstruction infiltrating macrophages are the prime source of the increased renal TGFβ production following ureteral obstruction. This concept is
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further supported by the observation that the kinetics of renal cortical macrophage infiltration correlate well with the increase in renal cortical TGFβ mRNA levels. Immunolabeling for intracellular TGFβ demonstrates that it is only detectable in the peri-tubular cells of the renal interstitium in obstructed kidneys, thus co-localising with the infiltrating macrophages [Diamond JR 1994]. Thus the release of proinflammatory cytokines such as TGFβ by macrophages can serve as an initiating mechanism to the overproduction of extracellular matrix by resident renal interstitial cells (e.g., fibroblasts) that will eventually cause interstitial fibrosis following obstructive nephropathy. However, it is also possible that beneficial effect of angiotensin converting enzyme inhibition could be in part explained by blocking a direct action of angiotensin II on intrarenal transcription of TGFβ. This is supported by the observation that whilst both angiotensin converting enzyme inhibition and angiotensin II receptor blockade reduce the degree of interstitial fibrosis and the increase in TGFβ following ureteral obstruction, only the former significantly ameliorates the macrophage infiltrate [Ishidoya S 1995]. These studies on the role of the renin angiotensin axis in modulating profibrogenic cytokine production provide an explanation at the cellular level for the observation that treatment with angiotensin converting enzyme inhibitors during prolonged (1-3 weeks) ureteral obstruction prevents subsequent loss of renal mass and hence preserves renal function [McDougal WS 1982].

The development of interstitial fibrosis following ureteral obstruction is accompanied by a phenotypic change of the interstitial fibroblast to a myofibroblast type (i.e. expressing α smooth muscle actin). Whole body X-irradiation so as to prevent the macrophage infiltrate not only lowers cortical interstitial macrophage
number, but also reduces cortical TGFβ and alpha-smooth muscle actin mRNA levels as well as the intensity of immunolabeling for alpha-smooth muscle actin. This observation further supports the concept that renal cortical TGFβ, derived from the infiltrating macrophage, in part, contributes to the subsequent interstitial fibrosis by fostering the modulation of fibroblasts to myofibroblasts within the renal cortex after ureteral obstruction [Diamond JR 1995b].

TGFβ also modulates the degradation of matrix proteins. A marked elevation of tissue inhibitor of metalloproteinase 1 (TIMP-1) mRNA expression (but not TIMP-2 or TIMP-3) has been found after unilateral ureteral obstruction. Inhibition of matrix protein degradation would enhance the accumulation of extracellular matrix proteins and further promote renal fibrosis in response to ureteral obstruction [Engelmyer E 1995].

### 9.5 Infiltrating cells following ureteral obstruction. Possible mechanisms for altered tubular cell function.

As well as altering renal function and promoting fibrosis, macrophage derived products have the potential to be able to modulate the function of tubular cells. A variety of immune cell derived cytokines have been shown to affect the activity of electrolyte transport systems in non-renal cells, raising the possibility that renal epithelia may also be subject to regulation by such factors. IL-1 is capable of stimulating amiloride sensitive sodium entry into a B cell line [Stanton TH 1986] and tumour necrosis factor (TNF) induces a decrease in the electrical potential across skeletal muscle plasma membrane within 5 minutes of initial exposure [Tracey KJ 1986]. In addition TNF also increases glucose transport into the muscle cell line L6,
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probably via an increase in the number of plasma membrane transporters [Lee MD 1987]. Both II-1 and TNF are macrophage derived products. Supernatants obtained from activated mouse peritoneal macrophages stimulate the sodium dependent uptake of glucose and aspartate in primary cultures of proximal tubular cells. Sodium independent transport processes are not affected by macrophage supernatants, suggesting that the factor is primarily acting by modulating sodium transport. Purification of the macrophage supernatant subsequently has shown that the active component is II-1 [Kohan DE 1988]. At least twelve hours of exposure to II-1 is required to directly increase sodium entry into proximal tubular cells, and both mRNA and protein synthesis are required for the effect.

II-1 also exerts a considerable, although somewhat different effect on distal nephron function causing a marked natriuresis and diuresis when infused into rats [Beasley D 1988, Caverzasio J 1987]. Micropuncture data have confirmed that this is a direct tubular effect with a threefold increase in sodium excretion being observed within one hour of II-1 infusion without a change in glomerular filtration rate, renal plasma flow or systemic blood pressure [Kohan DE 1989a]. Absolute delivery to the late proximal and mid distal tubule are unaltered by II-1 whereas sodium delivery to the papillary collecting duct is increased. This would suggest that II-1 is acting on the late distal tubule and collecting duct. The diuretic action of II-1 appears to be through the stimulation of the cyclo-oxygenase pathway since the effect is abolished by indomethacin [Beasley D 1988]. In addition, II-1 infusions induce a prompt rise in renal prostaglandin E₂ excretion, and II-1 increases prostaglandin E₂ synthesis by papillary collecting duct cells in culture [Kohan DE 1989b], but not by proximal tubular cells [Kohan DE 1990]. More recently II-1 has been shown to inhibit the Na⁺-

How the concentrations of cytokines used in these in vitro experiments (both cell culture and whole animal) relates to the in vivo situation is unclear. Since it is envisaged that the cytokines act in a paracrine fashion the local concentration of the cytokines will be substantially higher than in blood. It therefore seems likely that the concentrations of cytokines used in vitro to demonstrate a biological effect are within the range which could be found in vivo around tubular cells in disease states. In addition the concentration of cytokines shown to have an effect on tubular cells match those known to be effective in other biological systems.

Since cytokines appear to have different effects at differing sites along the nephron the localization of any infiltrate, as well as the products generated will determine the overall biologic effect. In view of the localization of the cellular infiltrate in obstructed kidneys around the distal tubule, it is tempting to speculate that eicosanoids and cytokines released from these cells may contribute to the alterations in tubular function seen in response to ureteral obstruction.
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Obstruction of urinary tract

Mechanical stimulation
Membrane stretch

Release of chemoattractant

Stimulation of intrinsic renal cells

Macrophage recruitment

Production of vasoactive substances and cytokines

Altered renal haemodynamics

Altered tubular function

Stimulation of fibrosis and sclerosis

Figure 9.1 Cascade of events leading to acute impairment of renal function and chronic structural damage in obstructive nephropathy.
9.6 Conclusion.

The effects of urinary tract obstruction are the result of variety of factors with complex interactions, resulting in alterations in both glomerular haemodynamics and tubular function as well as potentially permanent structural damage. Infiltrating macrophages appear to play a pivotal role in the modulation of these processes.

The application of these observations to clinical medicine remains to be determined. The understanding of the pathophysiological changes demonstrated in animals does hold the potential for developing effective therapies i.e. thromboxane synthetase inhibitors/receptor blockers, angiotensin converting enzyme inhibitors, antagonists of cytokines, and inhibitors of macrophage infiltration, for both hastening the recovery of renal function following the relief of obstruction and limiting any permanent damage to renal tissue. To date, however, the value of such therapies to human disease must remain speculative and the best treatment option remains the prompt and effective relief of the obstruction.
APPENDIX 1

INULIN ASSAY [Fuhr J 1955]

Reagents

1. Anthrone: 1.0g anthrone in 500ml 70% H$_2$SO$_4$.
   1.0g of anthrone was added to 300ml of 70% H$_2$SO$_4$ and allowed to dissolve with occasional stirring. The volume was adjusted to 500 ml and the mixture filtered. It was stored in a brown glass container for up to 1 week at room temperature.

2. 70% H$_2$SO$_4$: In a fume cupboard, 1400 ml of concentrated H$_2$SO$_4$ was added slowly to 600ml of distilled water.

3. 10% trichloroacetic acid (TCA): 20g of TCA was added to 200ml of distilled water.

Standards

A stock solution of 0.1g D-Fructose dissolved in 100ml distilled water was prepared. This was prepared fresh for each assay. The following standards were used:

- 1mg%: 1ml stock + 99ml distilled water
- 3mg%: 3ml stock + 97ml distilled water
- 5mg%: 5ml stock + 95ml distilled water
- 7mg%: 7ml stock + 93ml distilled water

Method

1. Precipitation of samples
   - 10µl plasma + 400µl 10% TCA
   - 100µl urine + 1000µl distilled water + 400µl 10% TCA

The sample was mixed well.
2. Analysis of samples

200μl of precipitated sample (blank, standard, plasma or urine) was added to 2.0ml of anthrone solution in a 12x75 Coleman cuvette and mixed well. This was then incubated at 55±1°C for 10 minutes. The samples were then allowed to cool to room temperature and % transmission read at 620μm. The concentration of inulin in the precipitated samples was then read off from the standard curve and the concentration of inulin in the plasma and urine calculated allowing for the dilution factor. All samples were run in duplicate. Results were expressed as mg%.
APPENDIX 2

PAH ASSAY [Smith HW 1945]

Reagents
1. 1 N Sulphuric Acid:
2. Cadmium Sulphate: 34.667g 3CdSO4.8H2O + 169.3ml 1 N H2SO4
3. 1.1 N NaOH: 44 g NaOH/l
4. 1.2 N HCl: 100 ml concentrated HCl/l
5. 100 mg% NaNO3: 0.1 g NaNO3 in 100ml distilled water.
6. 500 mg% Ammonium Sulphamate: 0.5g ammonium sulphamate in 100ml distilled water.
7. 500 mg% N-(1 naphthyl) ethylene diamine dihydrochloride: 2.5g in 500 ml distilled water.

Standards
A stock solution of 500 mg % was made by diluting PAH (Merck, Sharp & Dohme, West Point, PA, USA). A working solution of 2 mg % was then prepared by diluting the stock solution 1:250.
The following standard were prepared:-
0.02 mg %: working solution diluted 1:100
0.16 mg % working solution diluted 2: 25
0.24 mg % working solution diluted 3: 25

Method
1. Precipitation of Plasma
100µl plasma was added to 1 ml H2O in 10 x 75 mm tubes add mixed well.
300 µl CdSO4 and 100 µl 1.1 N NaOH were then added with further mixing.
The solution was centrifuged at 2500-3000 RPM for 15 minutes.

2. **Dilution of Urine**

The objective was to get the PAH concentration in urine < 0.24 mg %. The following dilutions were typically required:-

- Normal rats: 1:1000 - 1:4000.
- Uraemic rats: 1:500

Dilutions made in distilled water.

3. **Analysis of samples**

The assay was performed in 12 x 100 mm disposable tubes in duplicate. 2ml of blanks, standards, plasma (diluted 1:1 with distilled water) and urine samples were added together with 400 µl of 1.2 N HCl, 200 µl 1.1 N NaNO₂. The solution was mixed well. After 3 to 5 minutes 200µl of 500mg% ammonium sulphamate was added and mixed well again. After a further 3 to 5 minutes 200µl of 500 mg% N-(1 naphthyl) ethylene diamine dihydrochloride reagent was added. After 10 minutes the % transmittance of the solution was read in Coleman spectrophotometer at 540 nm wavelength. The PAH concentration of the unknown samples was read from semi-log plot of known standards with correction for the dilution. Results were expressed in mg%.
APPENDIX 3

RADIO-IMMUNO ASSAY FOR URINARY PROSTANOIDS

[Reginold DF 1981]

Reagents

1. Assay buffer: 0.9% NaCl, 0.01M EDTA, 3% bovine gamma globulin, 0.005% Triton X-100, 0.05% sodium azide in 50mM phosphate buffer at pH 6.8. For the PGE₂ assay 1% bovine serum albumin was also added.

2. The PGE₂ and TxB₂ antisera were prepared in the laboratory at Washington University St Louis MO, USA from rabbits. Their cross-reactivities and details of the RIA have been reported previously [Benabe JE 1982, Shayman JA 1985]. PGE₂ and TxB₂ antisera were used diluted 1:133 and 1:1000 in assay buffer respectively.

3. Radioligand: ¹²⁵I histamine-TxB₂ and ³¹I histamine-PGE₂. This was diluted in buffer to produce 10,000 counts per 100µl (typically 5µl in 10ml).

4. PEG 16% (w/v) in 50mM phosphate buffer at pH 6.8.
**Assay procedure**

The assay was performed in 12x75 siliconized tubes.

**PGE$_2$ standard curve and assay was constructed as follows:**

<table>
<thead>
<tr>
<th>Number</th>
<th>Dose</th>
<th>µl STD</th>
<th>µl buffer</th>
<th>µl Ab</th>
<th>µl label</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>TC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>blank</td>
<td>-</td>
<td>200</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>trace</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>2.5pg</td>
<td>2.5</td>
<td>97.5</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>5pg</td>
<td>5</td>
<td>95</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>10pg</td>
<td>10</td>
<td>90</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>20pg</td>
<td>20</td>
<td>80</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>30pg</td>
<td>30</td>
<td>70</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>50pg</td>
<td>50</td>
<td>50</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>100pg</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

50µl of the sample was diluted with 50µl of buffer in duplicate. 100µl of antibody and 100µl of label were then added.

**TxB$_2$ standard curve and assay was constructed as follows:**

<table>
<thead>
<tr>
<th>Number</th>
<th>Dose</th>
<th>µl STD</th>
<th>µl buffer</th>
<th>µl Ab</th>
<th>µl label</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>TC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>blank</td>
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<td>200</td>
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</tr>
<tr>
<td>3</td>
<td>trace</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>1pg</td>
<td>10(1:1000)</td>
<td>90</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>2pg</td>
<td>20(1:1000)</td>
<td>80</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>5pg</td>
<td>50(1:1000)</td>
<td>50</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>10pg</td>
<td>10(1:100)</td>
<td>90</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>20pg</td>
<td>20(1:100)</td>
<td>80</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>50pg</td>
<td>50(1:100)</td>
<td>50</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

25µl of the sample was diluted with 75µl of buffer in duplicate. 100µl of antibody and 100µl of label were then added.

The samples were incubated for 16 hours at 4°C. 2ml of cold 16% PEG was then added to each tube (except for the total counts - TC). Each tube was vortexed and allowed to sit for 30 minutes, when it was centrifuged at 3000 RPM for 30 minutes. The supernatant was decanted and as much of the PEG blotted from the tubes as possible. The tubes were then counted on the γ counter. %B/B$_0$ was calculated and a standard curve constructed by plotting %B/B$_o$ against log of known
standard. Concentrations of unknowns were read from the standard curve. The correlation co-efficient for the standard curve was typically >0.98.

A typical PGE$_2$ standard curve is shown below. In this case $r=0.99$.

![Graph showing standard curve](image)

**PREPARATION OF RADIOLIGAND.**

**Reagents**

1. Chloramine T (25 mg in 5 ml 0.05 M phosphate)
2. Na Metabisulphite (25 mg in 5 ml 0.05 M phosphate)
3. Methanol, distilled water, 15% ethanol, 95% ethanol and petroleum ether.

**Method**

The following were added to a siliconized 12 X 75 tube:

- 3μl of undiluted conjugate, 20 μl 0.5 M phosphate buffer, 5μl I-125 (0.5 mCi),
- 20μl Chloramine T. After 10 sec 30μl of Na Metabisulphite and 500μl water
were added.

The label was then purified on a Sep Pak by elution with 2 ml 95% ethanol (into siliconized tube) and the incorporation was checked (0.5 mCi = 500 million cpm)

The label was then separated by thin layer chromatography (TLC) in using chloroform:methanol:water = 90:10:1 as solvent and autoradiographed for 5-15 minutes. The proximal hot band was scraped (previous working label was also run on TLC to confirm position) and eluted in 95% ethanol in a siliconized tube and stored at -20° C until use.
Appendix 4

Lowry Assay [Lowry OH 1951]

Reagents

1. Lowry solution 2 ml 0.1 M NaOH
   50 ml 2 % Na₂CO₃
   1 ml 1 % CuSO₄
   1 ml 2 % NaK Tartrate 4H₂O
   This was prepared fresh for each assay.

2. Folin & Ciocalteu's Phenol Reagent  Diluted 2 N stock 1:1 in distilled H₂O

3. BSA standard (1 µg/µl)

Method

The assay was carried out in 12x75 glass tubes. 200 µl of standard or appropriately
 diluted sample was added. A standard curve was constructed of 0, 10, 20, 30, 40, 50,
 60, 70 µg BSA were made up to 200 µl in distilled water.

1 ml of Lowry solution was added and the mixture vortexed and allowed to stand for
10 minutes. 100 µl of Folin & Ciocalteu's Phenol Reagent was then added and the
  tubes vortexed. After 30 minutes absorbance at 750nm was measured in a Zeiss
spectrophotometer. Standards and samples were assayed in duplicate. A linear
standard curve was constructed and the unknowns read from the equation of the
regression line.
APPENDIX 5

WHITE CELL COUNTS [Berg WN 1945]

Reagents and Specimen

1. Turks solution 3ml of glacial acetic acid and 1ml of 1% aqueous solution of gentian violet made up to 100ml in distilled water.

2. Blood was collected in EDTA anticoagulant.

Procedure

1. Blood was diluted 1:20 in Turks solution mixed well.

2. The haemocytometer chamber was filled.

3. Cells were allowed to settle for 3 minutes.

4. Under high powered magnification the cells in the four large corners of the haemocytometer were counted (each large square contains 16 small squares). Cells overlapping the edges of the square were counted if they overlapped the left or upper borders, but not the right or lower borders.

Calculation

\[
WCC\ (10^9/l) = \frac{\text{cells counted} \times \text{dilution factor}}{\text{volume counted (nl)}} = \frac{\text{cells counted} \times 20}{\text{number of large squares} \times 100}
\]
DIFFERENTIAL WHITE CELL COUNTS

Procedure

1. A blood film was prepared on glass slide.

2. The smear was stained using Dif-Quik stain set according to the manufacturer's instructions (American Scientific Products II, USA).

3. Under high power magnification cell types were identified according to their staining characteristics as follows.

   - **Neutrophils**
     - Nucleus - dark blue
     - Cytoplasm - pale pink
     - Granules - reddish lilac

   - **Eosinophils**
     - Nucleus - blue
     - Cytoplasm - blue
     - Granules - red to orange

   - **Basophils**
     - Nucleus - purple or dark blue
     - Granules - dark purple

   - **Monocytes**
     - Nucleus (lobated) - violet
     - Cytoplasm - sky blue

   - **Lymphocytes**
     - Nucleus - violet
     - Cytoplasm - dark blue

4. The absolute differential cell count was calculated as:

   \[
   \text{total WCC} \times \frac{\text{percentage of a particular cell type}}{100}
   \]
REFERENCES


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REFERENCES


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REFERENCES


REFERENCES


-195-
REFERENCES


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