RABIES: PRE- AND POST-EXPOSURE STUDIES WITH HUMAN DIPLOID CELL STRAIN VACCINE

By

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Summary

This thesis studies the efficacy of human diploid cell strain rabies vaccine (HDCSV) in man. After a definition of rabies and a historical introduction a searching critical review is presented of all published work on the epidemiology of rabies, the virus, its replication, transmission and the pathogenesis; of human rabies, its pre- and postexposure prophylaxis; the adverse effects of treatment, and an assessment of immunity. The review reveals a desperate need for improved methods of treatment, setting the scene in 1975 when the studies began. Following a description of the volunteers, materials and methods is an account of rabies virus interference and the interference inhibition test which was developed as an alternative to the mouse neutralisation test (MNT). Restrictions in the use of rabies virus led to the rabies enzyme-linked immunosorbent assay (ELISA) described and used in later studies.

Studies 1, 2,  9 and 11 show that HDCSV is well tolerated by the intramuscular and intradermal routes and that both reliably induce high antibody titres, irrespective of age, sex, vaccine batch, ambient temperature, and passive immunisation. Studies 3 - 6 show that primary immunisation, not boosters, generally induces circulating interferon and that the interferon and humoral responses are poorly correlated. Studies 7 and 8 reveal a T-cell-mediated blast-transformation response in most vaccinees. Studies 9 and 10 show that satisfactory seroconversion rates are possible in busy centres using the intradermal route and that once only, postexposure, intradermal administration of 4 x 0.2ml HDCSV protects rabbits from street rabies virus. Studies 12 - 14 evaluate abbreviated candidate schedules of immunisation in Thais, one - a multisite intradermal schedule - was given to 78 patients bitten by rabid animals. A further 77 patients were treated with Semple vaccine. All patients survived. In conclusion, HDCSV is considered safe, thermostable and antigenic, and the intradermal route provides effective economical alternatives to the intramuscular route for both pre- and postexposure prophylaxis.
## Contents

<table>
<thead>
<tr>
<th>Page No.</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Acknowledgements</td>
</tr>
<tr>
<td></td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Declaration of work done by author</td>
</tr>
<tr>
<td></td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Publications arising from this work</td>
</tr>
<tr>
<td></td>
<td>20</td>
</tr>
</tbody>
</table>

### A. Background and historical review

#### Chapter 1. Definition and historical introduction

1. Definition .................. 24
2. Rabies before Louis Pasteur .................. 24
3. The Pasteurian Era . .................. 25
4. Rabies After 1885 .................. 26

#### Chapter 2. Global epidemiology of rabies

1. Introduction .................. 28
2. Species Involvement .................. 28
3. Rabies in Europe .................. 29
4. Rabies in the Americas .................. 32
5. Rabies in Africa .................. 33
6. Rabies in Asia .................. 36
7. Epidemiology of rabies in man .................. 37

#### Chapter 3. Rabies and rabies-related viruses

1. Introduction .................. 39
2. Virus structure .................. 39
3. Virus replication .................. 41
4. Rabies serotypes and related viruses .................. 42
5. Effects of physical and chemical agents .................. 43
Chapter 4. Transmission and pathogenesis
1. Transmission ................................................................. 44
2. Pathogenesis ................................................................. 45

Chapter 5. Human rabies; clinical features, diagnosis, and treatment
1. Factors influencing infection ........................................... 49
2. Incubation period ......................................................... 49
3. The prodrome .............................................................. 50
4. Patterns of disease ....................................................... 51
5. Complications ............................................................. 53
6. Differential Diagnosis .................................................. 55
7. Diagnostic Testing ....................................................... 57
8. Pathology and management .......................................... 60

Chapter 6. Pre- and post-exposure prophylaxis and assessment of immune status
1. Wound treatment .......................................................... 62
2. Neurotissue vaccines prepared from adult brain ............... 63
3. Neurotissue vaccines prepared from immature brain ......... 67
4. Avian tissue vaccines .................................................... 68
5. Tissue culture vaccines ................................................ 71
6. Passive immunization ................................................... 76
7. Assessing immunity to rabies ......................................... 81

B. The studies

Chapter 7. Subjects, materials and methods

Subjects & Materials
1. Subjects ........................................................................ 90
2. Vaccines ....................................................................... 91
3. Human rabies immune globulin (HRIG) ....................... 92
4. Equine antirabies serum (EARS) ................................. 93
5. Tissue culture materials .......................................................... 93
6. Buffers ................................................................................... 94
7. Tissue culture media .............................................................. 95
8. Cell cultures and their maintenance ..................................... 98
9. Virus diluents ......................................................................... 99
10. Cell fixative .......................................................................... 100
11. Cell stains ............................................................................ 101
12. Virus strains ......................................................................... 101
13. Reference preparations ........................................................ 103
14. Animals ................................................................................. 104
15. Solutions and Reagents for ELISA ........................................ 105

Methods
1. Mouse neutralization test ..................................................... 106
2. Assay of interferon ............................................................... 107
3. Identification of interferon .................................................... 108
4. Interference inhibition test .................................................... 109
5. Immunofluorescent staining ................................................ 112
6. Rabies enzyme-linked immunosorbent assay (ELISA) .......... 113
7. Comparative studies of vaccine potency by ELISA ............... 120
8. Lymphocyte transformation .................................................. 120

C. Results

Chapter 8. Rabies virus interference and the interference inhibition test
1. Development of the interference inhibition test (IIT) .......... 123
2. Assessment of observer error ............................................... 125
3. Reproducibility of the interference inhibition test ............... 125
4. Comparison of the interference inhibition test and MNT ...... 128
5. Comparative studies of interference ..................................... 130
6. Interference, immunofluorescence, and rabies virus yield ..... 133
7. Interference, interferon, and rabies virus yield .................... 134
8. Discussion ............................................................................ 136
Chapter 9. Enzyme-linked immunosorbent assay for the measurement of rabies antibodies

1. Introduction ............................................. 139
2. Development of the assay ......................... 140
3. Reproducibility of the ELISA test .............. 142
4. Comparison of ELISA and MNT ............... 142
5. Discussion .............................................. 143

Chapter 10. Studies with human diploid cell strain rabies vaccine and human rabies immune globulin

1. Introduction ............................................. 145
2. Study 1 - A trial of pre-exposure prophylaxis with HDCSV .......... 147
3. Study 1 - Clinical reactions to vaccination .......... 149
4. Study 1 - Antibody responsiveness: effect of age, sex, and vaccine batch ............................................. 161
5. Study 1 - Antibody response to primary immunization .......... 163
6. Study 1 - Antibody response to booster immunization .......... 165
7. Study 1 - Rapidity of booster responses .......... 167
8. Study 1 - Antibody titres at 36 months .......... 168
9. Study 1 - Discussion ...................................... 168
10. Study 2 - Human rabies immune globulin (HRIG) and postexposure immunization. .......... 172
11. Study 2 - Serological results ...................... 174
12. Study 2 - Discussion ...................................... 178
13. Studies 3 to 6 - Interferon response to human diploid cell strain rabies vaccine in man .......... 180
14. Titration of interferon ...................................... 181
15. Study 3 .............................................. 183
16. Study 4 .............................................. 183
17. Study 5 .............................................. 184
18. Study 6 .............................................. 186
19. Studies 3 - 6, Discussion ......................... 189
20. In-vitro transformation of lymphocytes from vaccinees .......... 191
21. Study 7, *In-vitro* transformation of lymphocytes following vaccination ........................................ 191

22. Study 8, Antibody production and *in-vitro* transformation of enriched T-lymphocytes following multisite ID vaccination ................................................................. 194

23. Studies 7 and 8, Discussion ........................................................................................................ 199

24. Study 9, Study of the responses to ID vaccination at British Airways Immunization Centre .......................................................... 202

25. Study 9, Discussion .................................................................................................................. 202

26. Study 10, Postexposure multisite ID administration of HDCSV in rabbits ......................................................... 203

27. Study 10, Discussion ................................................................................................................. 204

28. Study 11 - Stability of HDCSV rabies vaccine at high ambient temperatures ...................................................... 204

29. Study 11 - Data ................................................................................................................................ 206

30. Study 11 - Discussion .................................................................................................................. 209

31. Study 12 - Assessment of economical regimens of HDCSV for post-exposure prophylaxis .......................................................................................................................... 212

32. Study 12 - Adverse effects of vaccination .......................................................................................... 214

33. Study 12 - Serological results ........................................................................................................ 215

34. Study 12 - Discussion ..................................................................................................................... 221

35. Study 13 - Multisite intradermal and multisite subcutaneous vaccinations ................................................................................................................................. 222

36. Study 13 - Adverse effects of vaccination ...................................................................................... 224

37. Study 13 - ELISA results ................................................................................................................ 225

38. Study 13 - RFFIT results ................................................................................................................ 229

39. Study 13 - Discussion ..................................................................................................................... 232

40. Study 14 - Trial of multisite intradermal immunisation with HDCSV for post-exposure rabies prophylaxis ............................................................................................................ 234

41. Study 14 - Results .......................................................................................................................... 235

42. Study 14 - Discussion ..................................................................................................................... 242

43. Study 15 - Agreement between the RFFIT and ELISA for rabies antibody ................................................................................................................................. 244
D. Conclusions

Chapter 11. Conclusions

1. Introduction ................................................. 250
2. General conclusions ....................................... 250

E. References .................................................. 259
## List of figures

<table>
<thead>
<tr>
<th>Fig.</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.1</td>
<td>Inhibition of VSV plaque formation in monolayers of GL-V3 cells by CVS-11 rabies virus.</td>
<td>110</td>
</tr>
<tr>
<td>7.2</td>
<td>Intercept of quadratic curve fitted to optical density (OD) values of dilutions of test sera, and the straight line fitted to the mean plus 2.5 standard deviations of the mean of OD values of appropriate negative controls.</td>
<td>115</td>
</tr>
<tr>
<td>8.1</td>
<td>Correlation between rabies neutralizing antibody titres obtained by independent observers using the interference inhibition test (IIT).</td>
<td>126</td>
</tr>
<tr>
<td>8.2</td>
<td>Correlation of neutralizing antibody titres in serum samples retitrated by the IIT.</td>
<td>127</td>
</tr>
<tr>
<td>8.3</td>
<td>Zone of two- and fourfold differences from the regression line correlating rabies neutralizing antibody measured by the IIT and MNT.</td>
<td>129</td>
</tr>
<tr>
<td>8.4</td>
<td>Percentage inhibition of VSV plaque count by monolayers inoculated 96h earlier with increasing $\log_{10}$ MOI($\text{LD}_{50}/\text{cell}$) of different strains of rabies virus.</td>
<td>131</td>
</tr>
<tr>
<td>8.5</td>
<td>Virus yield from monolayers inoculated with $0.5 \log_{10}$ dilutions of ERA and CVS-11.</td>
<td>135</td>
</tr>
<tr>
<td>10.1</td>
<td>Predominant symptoms occurring within 10 days of primary ID vaccination and an ID booster following primary ID vaccination.</td>
<td>155</td>
</tr>
<tr>
<td>10.2</td>
<td>Regression of age against the antibody titre (IU/ml) 28 days after the first ID or IM injection with batches S0203 and S0322.</td>
<td>162</td>
</tr>
<tr>
<td>10.3</td>
<td>Antibody response to 0.1ml HDCS vaccine given ID and 1.0ml given IM on days 0, 3, 7, and 14, compared with the effect of 20 IU/kg Lister HRIG given on day 0 with ID vaccine therapy and IM vaccine therapy.</td>
<td>176</td>
</tr>
</tbody>
</table>
Fig. 10.4 Antibody levels following passive administration of 20 IU/kg Lister HRIG only and with 1.0ml quantities of HDCSV given on days 0, 3, 7, and 14 are shown in the left hand panels. The titres following administration of Cutter HRIG only, and with 1.0ml quantities of vaccine are shown on the right.

Fig. 10.5 Percentage reduction in VSV plaque counts following pre-treatment of GL-V3 cells with 1 unit of MRC Research Standard B, 69/19, human leucocyte interferon.

Fig. 10.6 GMT of rabies virus neutralizing antibody (IU/ml) of volunteers immunized with HDCSV. Left, comparison of titres in groups given vaccine alone and vaccine plus HRIG on day 0; right, comparison of titres in groups given 1.0ml vaccine ID in 8 sites and IM in a single site.

Fig. 10.7 Time course of individual and mean interferon responses to HDCS rabies vaccine in unprimed vaccinees.

Fig. 10.8 Individual and geometric mean titres of rabies neutralizing antibody after inoculation with needle and syringe and dermojet injector.

Fig. 10.9 Individual and geometric mean titres of rabies IgG antibody after inoculation with needle and syringe and dermojet injector.

Fig. 10.10 Geometric mean rabies IgG antibody titres after intradermal and intramuscular vaccination with HDCSV at Peshawar, Hyderabad, and Islamabad.

Fig. 10.11 GMTs following multisite ID regimens for HDCSV; evaluation of the response to second injections given either on day 7, or day 14.

Fig. 10.12 GMTs following multisite injections with HDCSV; comparison of the responses to 4, or 8 day 0 injections either administered ID, or SC with aluminium hydroxide adjuvant.

Fig. 10.13 Comparison of GMTs raised by a 4-site SC regimen and an 8-site ID regimen for HDCSV.
Fig. 10.14  Geometric mean titres (with 95% confidence limits) of antibody responses to HDCSV regimens without HRIG. Numbers at the base of some columns indicate number of patients with no detectable antibody.

Fig. 10.15  Geometric mean titres (with 95% confidence limits) of antibody response to HDCSV regimens with HRIG.

Fig. 10.16  Study 14. Geometric mean titres (with 95% confidence limits) of antibody response to rabies vaccine regimens.

Fig. 10.17  Regression plots of log$_{10}$ neutralising antibody and IgG antibody titres.

Fig. 10.18  Regression plots of log$_{10}$ neutralising antibody and IgG antibody titres.
List of tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 7.1</td>
<td>Titration of the international standard antiserum by the interference inhibition test</td>
<td>111</td>
</tr>
<tr>
<td>Table 8.1</td>
<td>Resistance to superinfection with VSV by monolayers infected with the CVS-11 strain of rabies virus</td>
<td>123</td>
</tr>
<tr>
<td>Table 8.2</td>
<td>Neutralisation of interference: incubation of CVS-11 with dilutions of the international standard antiserum for rabies for 1 hour</td>
<td>124</td>
</tr>
<tr>
<td>Table 8.3</td>
<td>Repeat titrations of the international standard antiserum in the interference inhibition test - dilution of the international standard causing a 50% reduction in VSV plaque counts</td>
<td>128</td>
</tr>
<tr>
<td>Table 8.4</td>
<td>Percentage inhibition of VSV at different times after infection with CVS-11 rabies virus.</td>
<td>132</td>
</tr>
<tr>
<td>Table 10.1</td>
<td>Population in Study 1 who were available for follow-up.</td>
<td>148</td>
</tr>
<tr>
<td>Table 10.2</td>
<td>Study 1; incidence of adverse reactions following primary ID vaccination on days 0, 28, and 56.</td>
<td>149</td>
</tr>
<tr>
<td>Table 10.3</td>
<td>Study 1; Symptoms occurring within 10 days of 204 intradermal injections given for primary immunisation.</td>
<td>150</td>
</tr>
<tr>
<td>Table 10.4</td>
<td>Study 1; Clinical signs 48 hours after primary intradermal injection.</td>
<td>151</td>
</tr>
<tr>
<td>Table 10.5</td>
<td>Study 1; Incidence of reactions after booster injections by the SC and ID routes.</td>
<td>152</td>
</tr>
<tr>
<td>Table 10.6</td>
<td>Study 1; Signs 48 hours after ID and SC boosters.</td>
<td>153</td>
</tr>
<tr>
<td>Table 10.7</td>
<td>Study 1; Symptoms occurring within 10 days of 50 intradermal boosters - 32 after 1° ID vaccination, 18 after 1° IM vaccination.</td>
<td>154</td>
</tr>
<tr>
<td>Table 10.8</td>
<td>Study 1; Symptoms occurring within 10 days of 172 intramuscular injections given for primary immunisation.</td>
<td>156</td>
</tr>
<tr>
<td>Table 10.9</td>
<td>Study 1; Clinical signs 48 hours after primary intramuscular injection.</td>
<td>157</td>
</tr>
</tbody>
</table>
Table 10.10 Study 1; Symptoms occurring within 10 days of 64 subcutaneous boosters - 35 after 1° ID vaccination, 29 after 1° IM vaccination.

Table 10.11 Study 1; Clinical signs 48 hours after ID and SC boosters

Table 10.12 Study 1; Summary of predominant symptoms after primary and booster injections.

Table 10.13 Study 1; Summary of reactions to primary and booster injections by the IM, ID, and SC routes.

Table 10.14 Study 1; antibody titres after primary immunization with HDCS rabies vaccine.

Table 10.15 Study 1; Effect of route and dosage of primary immunization on the antibody response measured 28 days after a booster dose.

Table 10.16 Study 1; Effect of route of administration and timing of booster doses on the antibody response measured 28 days after the booster doses.

Table 10.17 Study 1; The rapidity of the antibody response to booster doses of vaccine.

Table 10.18 Study 2; Schedules of sero-vaccine therapy.

Table 10.19 Study 2; Relation of dose of HRIG to mean weight, age, and sex of groups receiving vaccine and HRIG ± vaccine.

Table 10.20 Study 2; Comparison of GMTs following immunization with 20IU/kg Lister and Cutter HRIG.

Table 10.21 Study 3; Interferon-like activity found after vaccination with 0.1 ml ID or 1.0 ml IM.

Table 10.22 Study 5; Titres of circulating antiviral activity in two subjects given 1.0ml of HDCSV at 8 separate ID sites.

Table 10.23 Study 6; Schedules of immunization and number of vaccinees in each study group.

Table 10.24 Study 7; Increments in lymphocyte transformation and titres of rabies virus-neutralizing antibody on day 30 for 10 volunteers vaccinated with a human diploid cell strain rabies virus vaccine, as compared with responses of unvaccinated controls.
Table 11.25 Study 8; Mitogen and antigen stimulation of peripheral blood lymphocytes obtained 0, 10, 14, 21, and 42 days after vaccination of ten volunteers with 0.8 ml of HDCSV.

Table 10.26 Study 11; Results of the laboratory potency before and after the field-test in man.

Table 10.27 Study 12; Vaccination regimens of subjects.

Table 10.28 Geometric mean and range of IgG rabies antibody responses to HDCSV and Semple vaccine.

Table 10.29 Geometric mean and range of IgG rabies antibody responses to HDCSV and Semple vaccine: HRIG was administered with the first dose.

Table 10.30 Geometric mean and range of rabies neutralising antibody responses to HDCSV and Semple vaccine.

Table 10.31 Geometric mean and range of rabies neutralising antibody responses to HDCSV and Semple vaccine: HRIG was administered with the first dose.

Table 10.32 Study 13; Vaccination regimens of subjects.

Table 10.33 Study 13; Effect of passively administered HRIG (40 IU/kg) on actively induced rabies IgG.

Table 10.34 Study 14; Distribution of bite-sites.

Table 10.35 Study 14; Details of animals and bites.

Table 11.36 Study 14. Geometric mean and range of rabies neutralising antibody responses to HDCSV and Semple vaccine with and without EARS.

Table 10.37 Study 14; Percentage of patients without detectable neutralizing antibody.

Table 10.38 Study 14; Percentage of patients without detectable IgG antibody.

Table 10.39 Study 15; Agreement between ELISA and virus neutralization (RFFIT) for sera either positive or negative by both methods.
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Declaration of work done by the author

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Publications arising from this work


A. BACKGROUND AND HISTORICAL REVIEW
CHAPTER 1.
DEFINITION AND HISTORICAL INTRODUCTION

1. Definition
Rabies is an acute infectious encephalomyelitis caused by a Lyssavirus, a bullet-shaped virus belonging to the Rhabdovirus family. It is distributed globally among a variety of warm-blooded animals, primarily the Canidae and Chiroptera. Infection occasionally occurs in man, the disease being characterized by a lengthy incubation period, a plethora of central nervous system disturbances often including hydrophobia, and inevitably by death. Classical Greeks knew rabies as "lyssa" or "lytta" meaning madness. The Latin word "rabies" comes from the Sanskrit "rabhas" denoting madness or rage.

2. Rabies before Louis Pasteur
The history of rabies extends far into antiquity, reference to human deaths from the bites of vicious dogs being made in the Eshnunna code of Mesopotamia dating 2300 BC (Wilkinson 1977). Aristotle in the 4th century BC made the first undisputed description of the disease and its mode of transmission, but it was not until the 1st century AD that Celsus first described hydrophobia as a symptom and wrote on the presence of "virus" (Latin-meaning slime or poison) in the saliva.

In 1769, Morgagni noted that the onset of clinical rabies was often preceded by parasthesiae at the site of the wound and he postulated that neural rather than haematogenous spread of the virus might have occurred (Wright, 1959). Many detailed accounts of the disease and of bizarre attempts to prevent it accompany the history of rabies into the early 19th century when first Zinke (1804) and then Magendie (1821) substantiated John Hunter's premise (1793) that the disease might be transmissible experimentally by inoculation of infectious saliva from
animals and man.

3. The Pasteurian Era

The nineteenth century witnessed a growing awareness of the neurotropic nature of the virus culminating in the 1880's with Pasteur's epoch-making experiments and post-exposure treatment. The principle of prophylactic inoculation with material attenuated in the laboratory had been established in Pasteur's laboratory in 1879 with the chance attenuation of a forgotten flask of chicken cholera culture left undisturbed for several weeks instead of 24 hours. Chickens inoculated with the culture not only failed to succumb but resisted subsequent infection with a virulent strain (Dubos, 1950). Incredibly within a few years, Pasteur and his colleagues succeeded in developing a vaccine against anthrax, attenuated by cultivation at high temperature, and one against swine erysipelas, attenuated by passage through rabbits. He was also carrying out important experiments on rabies.

The clinical features and histologic changes of rabies led Pasteur to declare: "the central nervous system and especially the bulb joining the spinal cord to the brain are particularly concerned and active in the development of the disease" (Pasteur et al, 1881). He then announced that his group had successfully transmitted rabies by inoculating brain with infected nervous tissue and that the incubation period was shortened accordingly. In 1885, after promising attempts at virus attenuation by intracerebral passage in monkeys (Pasteur et al 1884), Pasteur described a new method of attenuation and its application to the first human post-exposure treatment (Pasteur, 1885).

By repeatedly passaging a bovine isolate through rabbits by intracerebral inoculation, Pasteur obtained a virus characterized by a shortened incubation period of 6 to 7 days and stereotyped clinical illness. He called this "fixed" virus, in contrast to natural infection caused by "street" virus. Pasteur's method of attenuation consisted of desiccating infected rabbit cords for periods of up to two
weeks. Dogs that were inoculated with extensively desiccated cord followed by injections of progressively less desiccated material resisted intracerebral challenge with virulent virus. More importantly, experiments made on dogs treated after rabid animal bites gave promising results (Pasteur, 1885).

Accordingly Pasteur felt justified to treat Josef Meister, a 9-year old boy from Alsace, who was bitten at 14 sites on the hands, legs and thighs by a rabid dog. The worst of the wounds were cauterized and on the evening of July 6th, 60 hours after the attack, the boy was inoculated with material desiccated for 15 days. Over the next 10 days he received a further 12 injections of increasingly less desiccated cord, ending with fully virulent virus. The boy survived without ill-effects and in October 1885 a second patient was treated. Pasteur's vaccine then became widely available in Paris and by November 1886 2 490 people had been treated.

4. Rabies After 1885

Remarkably little was known of the disease agent until the early 20th century when Negri first visualised darkly staining inclusions in certain nerve cells in the brains of rabid animals (Negri, 1903). He wrongly considered the inclusions to be protozoan, nonetheless, his discovery was of major diagnostic importance. The year 1903 also saw Remlinger's filtration experiments establish the agent to be of ultramicroscopic proportions (Remlinger, 1903), a finding confirmed soon afterwards by centrifugation studies (Barrat, 1904; Remlinger, 1905). A further 30 years elapsed, however, before ultrafiltration analysis suggested a value of 100-150 nm for the particle diameter, a reasonable approximation of today's accepted figure (Galloway & Elford, 1936).

In 1913, Noguchi and Levaditi reported independently that rabies virus could be maintained in fragments of neural tissue (Noguchi, 1913; Levaditi, 1913), though in neither case was the virus passaged into explants from non-infected animals. No further progress with in vitro cultivation was made until the 1950's
when the virus was first grown (Bequignon et al, 1954) and then serially propagated (Kissling, 1958) in cells of non-neural origin. This was soon followed by demonstrations of its growth in a variety of primary and continuous culture systems, not only in cells from warm-blooded animals, but also in several established cell-lines of poikilothermic vertebrate origin. The 1950's also saw the virus adapted to grow in avian embryos (Powell & Culbertson, 1950), but it was exploitation of cell culture techniques that proved the most helpful in establishing many of the physicochemical, biologic, and antigenic properties of the virion.

Within a few years much was learned of the virus. The antigen content of Negri bodies was identified by several immunological techniques including immunofluorescence (Goldwasser & Kissling, 1958), ferritin labelling and electron microscopy (Atanasiu et al 1963), and immunoperoxidase (Atanasiu et al 1971). Elongated particles, which were thought to be virus were first visualised by electron microscopy of nerve cells in 1962 (Matsumoto, 1962) and in 1963 attention was drawn to their bullet-shape and similarity to vesicular stomatitis virus, the first rhabdovirus described (Davies et al 1963). Other reports in 1963 concerned the virion nucleic acid; the red colour of rabies antigen when stained with acridine orange, the replication of virus in the presence of DNA inhibitors, and its inhibition by 5-fluorouracil all suggested rabies to be an RNA virus (Hamparian et al, 1963; Kissling & Reese, 1963; Lépine & Atanasiu, 1963).
CHAPTER 2.
GLOBAL EPIDEMIOLOGY OF RABIES

1. Introduction
Rabies is enzootic in all continents except Australasia and Antarctica. Most other disease-free areas are islands or less commonly peninsular land masses where stringent quarantine regulations can be enforced. All warm-blooded animals are susceptible to the virus including birds though these are rarely if ever infected in nature. The disease exists in two epidemiologic forms; urban rabies which is propagated chiefly in feral and domestic dogs and is prevalent in many developing countries of the world; and sylvatic rabies which occurs in a wide range of species, principally small carnivores and Mustelids.

2. Species Involvement
According to the 1981 WHO survey (WHO, 1982a) and figures from the United States (MMWR, 1982), rabies is most prevalent among wild canids (foxes, wolves, and jackals 35.5% of 38,089 cases), followed by dogs (33.8%), skunks (11.8%), farm animals (7%), cats (3.8%), bats (2.3%), mongooses (0.3%), and other species (5.5%). Extensive surveys show rodent rabies to be extremely uncommon (Horrenburger, 1952; Winkler, 1972; Bögel et al, 1975, 1976; Förster et al 1977; Botros et al 1979; Steck & Wandeler, 1980; Summa et al, 1987). Rabies is unusual among lagomorphs (i.e. rabbits and hares) and insectivores (i.e. shrews, moles, and hedgehogs), though Mustelids (i.e. skunks, weasels, badgers, and martens) and the Viverridae (i.e. mongoose, suricate, ferret, genet, civet cat, and polecat) are regularly found infected (Winkler, 1972; Bögel et al, 1975; Förster et al 1977; WHO, 1982a; CDC, Oct 1981).
Non-human primates are occasionally infected and isolated cases have been reported throughout Africa, South America, and parts of Asia (Bisseru, 1972; WHO, 1977). Among the wild Felidae, sporadic cases have been identified in lions, hyaenias, leopards, cheetahs, lynxes, tigers and ocelots (Bisseru, 1972). Rabies among wild ungulates is regularly identified in central European deer (Bögel et al, 1975; WHO, 1977; Steck & Wandeler, 1980; WHO, 1980a), and is recorded in camels in North Africa and the Near- and Middle-East (Bisseru, 1972; WHO, 1976); kudu and eland antelopes in South-West Africa (Schneider, 1985); and occasionally in buffalo and moose in North America (CDC, Oct 1981).

The Chiroptera are of major importance as reservoirs and transmitters of infection in the Americas. In Latin America bites by haematophagous vampire bats cause losses of more than 100,000 cattle each year (Acha & Arambulo, 1985), and in the United States bats represent the most widely distributed vector. In Europe and Asia, bat rabies has been reported but seems very rare (Nikolic’ & Jelesic, 1956; Pitzschke, 1965; Smith et al 1967; Pal et al 1980).

3. Rabies in Europe

Democrates in 500 BC and Aristotle in 322 BC mention rabies in Greece almost 2,500 years ago. The earliest suggestion of rabies in Britain is in the Laws of Howel the Good of Wales in the year 1026 when reference was made to an outbreak of canine madness (Smithcors, 1958). In Europe throughout the Middle Ages, rabies was widely prevalent among wild dogs and wolves and was transmitted occasionally to domestic animals and man (Steele, 1975). By 1613, it caused sufficient alarm for Spackman to publish the first English work dealing exclusively with the disease (Barber-Lomax, 1960). In 1684 and 1708 two human cases were reported to the Royal Society following attacks by rabid foxes (Howman, 1684; Mead, 1708). Both accounts omit to relate whether or not the foxes were wild and free-living, but rabies in foxes in continental Europe was recognized as long ago as 1271 in France.
During 1759 and 1760 rabies was sufficiently well established in London that in An Essay on the Bite of a Mad Dog, Layard in 1763 states that magistrates ordered all dogs to be confined within doors for a month, and officers were empowered to destroy all dogs found at large (Smithcors, 1958). Persistence of rabies in Britain, especially in the heavily populated urban areas in the Midlands (MAFF, 1965), led in 1831 to a Bill to Prevent the Spreading of Canine Madness in attempts to control it. In 1886, rabies was made a notifiable disease under the Contagious Diseases (Animals) Act, and the Rabies Order of 1887 gave local authorities powers to muzzle, control, seize, detain and dispose of stray dogs. The poor enforcement of this legislation by local authorities led to the introduction of a new order in 1890, the Rabies (Muzzling of Dogs) Order, which brought the disease under central control and achieved considerable success. Due to public opposition to muzzling this order was revoked in 1892. The position deteriorated and within 3 years the number of cases in dogs increased almost 20-fold to an all-time peak of 727 cases (Hill, 1971).

A new Rabies Order and the first Importation of Dogs Order came into operation in 1897 and, with vigorous enforcement, they succeeded in eradicating the disease from Britain in 1902, it apparently having never been established in wildlife. The country remained rabies-free until 1918 when it was reintroduced by a dog illegally imported at Plymouth. Altogether 328 cases were confirmed before the disease was again eradicated in 1922. Since then only two cases of have occurred outside quarantine (Hill, 1971).

In northern and western Europe, dog rabies virtually disappeared at the turn of the century, possibly through the stray dog problem being brought under control, and the epizootic among foxes also receded (Lloyd, 1976). The origins of the present epizootic are not known. The disease was first noted in foxes in Poland in 1939-40; it penetrated Germany and Czechoslovakia in 1950, reached Denmark in 1964, Belgium and Luxembourg in 1966, Switzerland in 1967, France in 1968, the
Netherlands in 1974, and Italy and Yugoslavia in 1977 (WHO, 1978a; Petrovic’ et al, 1980). The frontwave of the epizootic has advanced south-westwards an average of 37 km per year, the annual shift varying between a temporary standstill and progression of 60-80 km (Steck & Wandeler, 1980), with progress being modified by wildlife population densities and surface topography (WHO, 1982b).

In newly invaded zones foxes account for more than 95% of all animals found rabid (Bögel et al, 1976). The frontwave epizootic usually lasts no more than one or two years. In its wake, there is a relative increase of rabies in other animals including badgers, martens, polecats, deer and domestic species which together account for some 20-30% of cases (Bögel et al, 1976; Steck & Wandeler, 1980). The frontwave is generally followed by 3-5 years of low incidence or absence of rabies due to drastic reduction in fox density, but reinvasion from adjacent endemic areas has occurred with the rapid recovery of fox populations (Wandeler, 1980).

In 1979, a total of 16,820 rabies cases were reported from 18 European countries participating in the European Rabies Surveillance Scheme (WHO, 1980a). No cases were reported from Bulgaria, Portugal, Scandinavia and the United Kingdom; a dog imported from India was the only case notified by the Netherlands; and a cat from North Africa was the only case reported by Spain (CDC, 1981). Animals most frequently reported rabid were foxes (70.1%), cattle (6.2%), deer (4.1%), mustelids (4.1%), and cats (4.0%). Dogs accounted for 7.7% of all cases when Turkey is included, but only 1.9% for all other countries. Four human cases were reported, two from Yugoslavia (Petrovic’ et al, 1980).

There is a high incidence of rabies in raccoon dogs in parts of the USSR, and foci of fox rabies are found in the Baltic republics, Byelorussia, the Ukraine and Kasachstan (Steck & Wandeler, 1980). Within the arctic circle and Greenland, rabies is prevalent in arctic foxes, reindeer, wolves and sledge dogs, and has been diagnosed in a seal (Müller, 1966; Johnson, 1971; Ødegaard & Krogsrud, 1981).
4. Rabies in the Americas

The middle of the 18th century marks the beginning of canine rabies in the northeastern United States (Johnson, 1959). It followed the trails of the early settlers and was recognized in California by 1836 (Bisseru, 1972). By the early 20th century, rabies had appeared in all but 9 states and was recorded in skunks and foxes. Bat rabies was first identified in the United States in 1953 (Venters et al, 1954). Since then 30 of the 39 species of bat considered residents of the continental United States and Canada have been reported rabid (Constantine, 1979).

In Mexico, the first documented outbreak of canine rabies occurred in 1709, initially in the vagrant dog population of Mexico City. It spread extensively throughout the ancient territories of New Spain and involved both cattle and man (Carrada, 1978). In the Mexican peninsular of Yucatan and in what is now known as Panama, vampire bats were evidently infected with rabies prior to the discovery of the New World, since many Spanish conquistadors and their livestock died with a rabies-like illness following bites by bats until effective remedies, consisting of washing the wound and cauterizing it with embers, were learned from the Indians (WHO, 1962). Although haematophagous bats were known to transmit disease to cattle in the 1500's, it was not until 1911 that Carini recognized it as rabies and identified Negri bodies in the brains of dead cows (Carini, 1911).

In Trinidad during the period 1929 to 1935, vampire bats were convincingly incriminated in an outbreak of paralytic human rabies involving 55 cases (Pawan, 1936). It has since been controlled by suitable public health measures. Elsewhere in the Caribbean, the first case of mongoose rabies was recognized in Puerto Rico in 1950 (Acha & Arambulo, 1985). Since then, mongoose rabies has been identified in Cuba, Dominica, Grenada, Puerto Rico and the U.S. Virgin Islands. From its introduction to the Caribbean in the 1870's for the purpose of rat control, the Indian mongoose is now the major terrestrial wildlife host in the region (Everard et al, 1981). In Grenada, the prevalence of mongoose rabies is
1.3%, and of 208 persons who received treatment during the 10-year period 1968 to 1977, 57% were bitten by mongooses (WHO, 1980b).

The current pattern of rabies in the United States and Canada is the reverse of that in Latin America where more than three-quarters of the reported 10,948 animal cases in 1981 were in dogs (Acha & Arambulo, 1985). In 1981, 7,211 laboratory-confirmed cases of animal rabies were reported from 48 states and Puerto Rico to the Centers for Disease Control for the United States and its territories (CDC, 1983). Seven species of animals accounted for 97% of the total reported cases: skunks (62%), bats (12%), raccoons (7%), cattle (6%), cats (4%), dogs (3%), and foxes (3%). Rabid bats were the most widely distributed species geographically, with confirmed cases in 46 states. Raccoon rabies, which had been reported only sporadically before 1950, is now endemic in the southeastern states and is spreading in areas in northern Virginia, West Virginia, and Maryland (MMWR, 1982). Rabid skunks are widely distributed, mostly in the Central and Pacific regions, and the majority of rabid foxes in the United States are localised to New York, Texas, Maine and Alaska.

Fox rabies is more prevalent in Canada than in the United States and of the 1,655 rabies cases in 1979 44.4% occurred in this species (CDC, Oct 1981). Skunks accounted for 25.7% of cases, cattle 13%, dogs 5.5%, cats 3.3%, bats 2.7%, and the remaining 5.4% of cases occurred in farm animals, wolves, raccoons and coyotes. More than 85% of cases were reported from the province of Ontario.

5. Rabies in Africa

Although the co-existence of rabies and all the serologically-related rabies-like viruses strongly suggest that the Lyssaviridae originate from this part of the world, there is little documentary evidence of rabies in sub-Saharan Africa before the 1900's. The treatment of bites by rabid animals was described in Ethiopian medical books in the early 17th century, and early 19th century travellers to Ethiopia reported the presence of rabies throughout the country (Fekadu, 1982). In 1887,
rabid dogs inflicted heavy losses amongst cattle and small-stock in South West Africa/Namibia (Schneider, 1985); in 1893, a dog imported from England was found rabid in Port Elizabeth, South Africa; human rabies was reported in Madagascar in 1896 and an Institut Pasteur was founded in 1898 (Rakotonirina-Randriambeloma & Coulanges, 1985); rabies was first recognized in the Sudan in 1904 (Ibrahim et al, 1985); and in Kenya laboratory tests established its existence in 1912 (Sionkok & Karama, 1985).

Although reliable information is sparse, the available data indicate that rabies is prevalent throughout most of Africa and the domestic dog is the main vector. In East Africa, rabies is endemic throughout the Sudan where 198 human cases were reported during the three-year period 1980 to 1982, mostly from Kassala Province (Ibrahim et al, 1985). In Ethiopia, 159 human cases were unofficially reported to the Central Veterinary Laboratory during the same period; 108 cases were reported from Shoa, 24 from Addis Abada, and, of the total, 97.5% were bitten by dogs (Ayalew, 1985). Rabies in Kenya has constantly been in two provinces, Coast and Eastern, and appeared in Rift Valley, Western, Nyanza and Central Provinces from 1979 (Sionkok & Karama, 1985). Of 390 cases examined from 1977 to 1982, dogs accounted for 60.5%, domestic species 26%, man 7.4% (29 cases), and wildlife species (mostly jackals, honey badgers and hyaenas) for 6.1%. In Tanzania all regions except the southern coastal region of Mtwara-Lindi and Zanzibar report rabies and 19 to 83 human cases were recorded annually during 1979-1982. Dogs accounted for 82% of the 164 confirmed cases between 1977 and 1981 and wild animals for only 3%; in 1958 and 1959 an appreciable number of human cases were associated with attacks by jackals (Magembe, 1985). Dogs are the principal vectors in Madagascar; of 1226 specimens found positive from 1959 to 1982, 1098 (91%) were canine, 74 (6%) bovine, 32 feline, 4 from other domestic species, and 18 cases occurred in man (Rakotonirina-Randriambeloma & Coulanges, 1985). The species distribution of cases in Mozambique, where 123
human cases were notified in the 5 years from 1978 to 1982, is similar to that in
other East African countries with dogs accounting for 85% and wildlife (the vervet
monkey and foxes) only 1.5% of the 317 confirmed cases from 1973 to 1982. Most
cases were located in the southern provinces bordering Swaziland and South Africa
(Dias et al, 1985).

From its virtual elimination between 1959 to 1964, rabies was confirmed
in more than 2000 animals in Zimbabwe during the 3-year period 1980 to 1982.
In one epizootic which began in 1979, rabies was carried 200 kilometres by jackals
in 2 years and was introduced into dogs and cattle; jackals accounted for 74% of the
680 animals confirmed rabid; and dogs and cattle 11% each (Foggin, 1985). In
Zambia, canine rabies is widespread with an average of 150 cases per year. Human
rabies occurs with an annual average of 21.4 cases mostly in Luapula Province and
Copperbelt Province where canine-human population densities are high. Bovine and
wildlife rabies (principally among jackals) are also recorded but are localised
mostly in the Central, Southern, and Lusaka Provinces (Röttcher & Sawchuk, 1978;
Zyambo et al, 1985). A similar pattern is seen in Botswana with a mean of 66
animal and 4 human cases per annum; Francistown, Ghanzi, Maha-Lapye, and
Gabarone are "high-risk" areas (Maganu & Staugard, 1985).

Sylvatic and urban rabies are both well recognized and widespread in South
West Africa/Namibia. The chief vectors are dogs in the northern districts, with dogs
and humans as main victims, and jackals in the central districts causing losses
amongst cattle and probably responsible for an outbreak in kudu antelope and wild
felidae in the south (Schneider, 1985). During 1982, there were only 256
laboratory confirmed cases of rabies, but a game census indicated a loss of 30,000 to
50,000 kudu. In South Africa rabies occurs frequently in the yellow mongoose
particularly in the grassveld zone of the central plateau, domestic and wild canids in
the northern Transvaal, and farm animals (Barnard, 1979; WHO, 1982a).
6. Rabies in Asia

Reference to human deaths from the bites of mad dogs was made in Mesopotamia in the Eshunna legal codes dating 2300 BC. Rabies has existed in India since Vedic periods, and the Latin word "rabies" seems to have originated from the Sanskrit word "rabhas" meaning to do violence (Ahuja et al, 1985). Rabies is a serious public health problem throughout Asia, though Bahrain, Japan, Singapore, Taiwan, Papua-New Guinea and the United Arab Emirates are rabies-free (Abdussalam & Botton, 1974; WHO, 1982a). Reports from Turkey indicate a high incidence of urban dog rabies and in 1979 there were 385 cases (24.1%) in cattle (WHO, 1980a). In Israel, rabies has declined in the northern region, but has penetrated the central area which had been practically free for at least 10 years, with the emphasis in wildlife (WHO, 1982a). Canine rabies occurs in rural Syria, Iraq, (Abdussalam & Botton, 1974) and Iran, especially in the north-eastern region where it also prevails in wolves, jackals and foxes (WHO, 1976; Fayaz, 1985).

Domestic dogs, chiefly strays, are largely responsible for the spread of rabies in Afghanistan and the Indian sub-continent. Foci of sylvatic rabies occur mostly in jackals, though other Canidae and Felidae are are sometimes involved, as are mongooses and occasionally bandicoots (Singh, 1980). In Pakistan, 54 horses and donkeys, 71 cattle and buffalo, 15 sheep and goats, and 393 dogs and cats were reported during a 4-year period (Lari, 1985). Rabies is prevalent in dogs cats and wildlife throughout Bangladesh (WHO, 1982a), and although most hydrophobia deaths in India are reported in Maharashtra, Tamil Nadu, West Bengal and Andra Pradesh, no mainland state is rabies-free (Ahuja et al, 1985).

Canine rabies is endemic in Korea with most cases being reported in the central region (Young-Hi, 1985). In Malaysia, rabies is confined to the border states with Thailand where more than 7,000 animal cases are confirmed annually, the majority occurring in domestic and stray dogs (97%), followed by cats, oxen, swine and occasionally wildlife (Thongcharoen & Wasi, 1985). Indonesia reported an average of 869 cases annually during the period 1978-1982; the predominant
species involved were dogs (95%), cats (3%), monkey and others (2%), with most cases in North and West Sumatra, West Java, and North Sulawesi (Koesharyono et al, 1985). Dogs are also the chief vectors and reservoirs of the virus in Burma, Cambodia, Laos, Viet Nam and the Philippines, which has been consistently among the countries with the highest human death rate from rabies in the world. Here the incidence is highest in densely populated coastal and central plains of the islands and lowest in sparsely populated central mountain areas (Beran et al, 1972). The distribution of rabies in China is uncertain, but cases are reported in both wild and domestic Canidae.

7. Epidemiology of rabies in Man

Official reporting of rabies in man is grossly deficient in many of the less developed countries so the true extent of the human disease is unknown. The available data must be interpreted with caution. In India, for example, 18 cases were reported in the 1975 survey of the World Health Organisation (WHO, 1977), yet 640 cases were reported in a few provinces (Pasteur Institute of Southern India, 1977). Similarly in Thailand the WHO survey records 17 cases, a figure far fewer than the 294 cases reported from Bangkok and provincial hospitals (Sinhaseni, 1979). Even these higher estimates are at best conservative, as many outlying villages do not report disease, although as many as three-quarters of the cases are believed to occur in rural areas (Singh, 1980). Data from other sources confirm that human rabies is a much larger problem than is officially recognized. In India, for instance, a survey of teaching hospitals showed an incidence of one case of human rabies per 2000 admissions, and in certain areas the incidence was four times higher (WHO, 1970). Thus from estimates of the total population and the numbers hospitalised, it is probable that at least 17 000 persons perish from rabies in India each year. This may still be an underestimate, since many cases never reach hospital, and, of those that do, many undoubtedly are misdiagnosed, either through inadequate diagnostic
facilities or atypical clinical presentation. San Martin et al (1967) discovered that 27 of the 1,596 people (1.7 per cent) on whom post mortems were carried out in Cali, Colombia, in 1962 had died of rabies, although only one or two cases had been diagnosed annually in years past. Similarly, 21 per cent of all cases in the United States between 1960 to 1979 were not diagnosed until after death, and may have gone undiagnosed if postmortem pathologic or virologic studies not been undertaken (Anderson et al, 1984).

Apart from the Indian subcontinent and South East Asia, hydrophobia is prevalent throughout much of Africa, South and Central America, and Mexico (WHO, 1977; Foggin & Swanepoel, 1979; Bennelmouffok et al, 1979; Fagbami et al, 1981; PAHO, 1981; Fekadu, 1982). Few tropical countries have rabies under control, and the risk of exposure in most developing countries is still formidable. Indeed, a recent survey of missionaries and foreign aid members working in the Third World showed that the overall risk, based on a mean stay of 4-5 years, was 16 per cent per household (Bjorvatin & Gundersen, 1980). More extensive analyses in 30 tropical countries show the incidence of rabies to be 0.1 to 25.8 cases per million population (mean, 3.7), and the number of treatments to be 2.7 - 4,570 (mean, 867) per million population (Bögel & Motschwiller, 1986). Dog bites are responsible for the majority of human cases and for virtually all post-exposure rabies vaccinations. Two-thirds to three-quarters of human exposures and cases occur in males, and persons aged less than 20 years are the most frequent victims (Fagbami et al, 1981; Trivedi, 1981).
CHAPTER 3.
RABIES AND RABIES-RELATED VIRUSES.

1. Introduction
Rabies virus is a member of the family Rhabdoviridae (rhabdo, Greek, meaning rod), a name referring to the bullet-shaped or bacilliform morphology of its members. Approximately 80 other bullet-shaped viruses have now been recovered from a variety of plants and animals, including fish and insects (Brown et al., 1979). The family Rhabdoviridae has two genera which infect invertebrates, the Vesiculovirus and Lyssavirus genera, with rabies and the serologically related Lagos bat, Mokola, Duvenhage, Kotonkan, and Obodhiang viruses belonging to the latter. Rabies isolates from naturally infected animals are generally referred to as "street" virus. In contrast, the term "fixed" was first applied by Pasteur in describing the highly reproducible biological properties which appeared after intracellular passage of street virus in rabbits; it is now also applied to viruses passaged in avian embryos or tissue culture and having characteristic biological behaviour.

2. Virus structure
Rabies virus is an enveloped RNA virus with average dimensions 180 x 75 nm (Hummeler et al., 1967). The particles are composed of a helical nucleocapsid enclosed in a bilaminar membrane envelope with spike-projections 6-7 nm long having knob-like structures at their distal end (Hummeler et al., 1967; Vernon et al., 1972). By electron microscopy it appears that the surface projection layer is hexagonally arranged. The protrusions are absent at the base, which is frequently invaginated forming a hollow axial channel. In addition to the typical virus particles, anomalous forms such as short, V- and Y-shaped virions have been
described (Hummeler et al, 1967; Murphy, 1975). Rabies defective interfering (DI) particles measuring 70-100 nm in length (Murphy, 1975) contain RNA and are antigenically similar to full-length particles. DI particles interfere with the replication of full-length virions (Kawai et al, 1975) but cannot replicate in their absence.

The gross chemical structure of rabies virus has been estimated at ~24 per cent lipids, ~3 per cent carbohydrates, ~1 per cent RNA, and ~72 per cent protein (Sokol, 1974; Schneider & Diringer, 1976). The virus contains five proteins, namely L, G, N, NS and M in order of decreasing molecular weight (Sokol et al, 1971; Madore & England, 1977; Dietzschold et al, 1979; Coslett et al, 1980; Crick, 1985). The single strand of RNA (Sokol et al, 1969) is intimately associated in the virion with about 1,800 molecules of phosphorylated nucleoprotein N (Madore & England, 1977) and loosely bound with proteins L and NS forming a nucleoprotein core. This is surrounded by a viral envelope which contains two non-glycosylated membrane proteins (M₁ and M₂) and an external lipid envelope through which the surface spikes (glycoprotein G) project (Sokol et al, 1971; Madore & England, 1977). The glycoprotein G, which is the only protein external to the virion, is required for virus infectivity (Sokol et al, 1969; Neurath et al, 1972). It is also responsible for the recognition of specific cell-surface receptors and induces and reacts with virus neutralising antibody and antibody which lyse infected cells in the presence of complement (Schneider et al, 1973; Wiktor et al, 1973a; Cox et al, 1977).

The RNA of this negative-strand virus is non-infectious (Sokol et al, 1969). It has a molecular weight of 3.8 - 4.6 x 10⁶ (Sokol et al, 1969; Holloway & Obijeski, 1980), a sedimentation coefficient of 45S, a buoyant density in Cs₂SO₄ of 1.66 g/cm³, and when uncoiled is about 4.2 μ long (Sokol et al, 1969).

The amino acid sequence of the glycoprotein G has recently been deduced for ERA (Anilionis et al, 1981) and CVS (Yelverton et al, 1983) strains of virus and
for a number of mutants (Dietzschold et al, 1983). Preliminary data indicate that more than one site may be involved in determining virulence (Coulon et al, 1983; Dietzschold et al, 1983). Antigenic characteristics of the protein have been studied by using mutants resistant to neutralisation by monoclonal antibodies. One major site recognised by around 70% of the available monoclonals, site II, has been described by Lafon et al (1983). The presence of another site (site III) is recognised by 20% of the monoclonals and several minor sites were also demonstrated. Site III, which extends from amino acids 330-34, is implicated in the recognition of specific receptors present on nerve endings (Seif et al, 1985), and mutations at amino acid 333 modify the host range spectrum of the virus (Kucera et al, 1985). Mutations at antigenic site II, especially those affected in amino acid 198, have reduced pathogenicity (Prehaud et al, 1988). The glycoprotein has also been fragmented and the fragments tested for their ability to evoke neutralising antibody synthesis and reactivity with virus-primed T-lymphocytes (Dietzschold et al, 1985). Here again it appears that specific immunologic functions are associated with different regions of the molecule.

3. Virus replication

Virus replication takes place in the cellular cytoplasm which the virus enters either by fusion of the lipid bilayer with the plasma membrane or by engulfment (Brown & Crick, 1980). The general scheme of rabies RNA transcription is believed to be similar to VSV and to proceed via the formation of a full-length positive RNA copy that is an intermediate of genome replication, and 5 polyadenylated complementary monocistronic messenger (m) RNAs (Holloway & Obijeski, 1980; Coslett et al, 1980). The L protein is believed to act first as the transcriptase responsible for synthesis of the mRNAs (Kawai, 1977). The NS protein may then be responsible for the addition of a poly (A) tract to the 3' end of cleaved mRNAs as has been proposed for VSV replication (Naito & Ishihama, 1976). The mRNAs attach to membrane-
bound polysomes or cytoplasmic ribosomes for synthesis of their respective protein molecules (translation) (Crick, 1983). The G protein is then glycosylated before virion assembly (Crick, 1983). In common with other negative-strand RNA viruses, the newly synthetized plus-stranded RNA eventually stops being generated into the form of multiple mRNA species; instead it serves as template to synthetize the progeny negative-stranded RNA molecules, the L protein probably acting as the replicase in such a reaction (Carrasco & Smith, 1984). Subsequently, the genomic RNA associates with N, NS, and L proteins to form the nucleocapsid which migrates to cell membranes, these being converted into virion envelope by insertion of proteins G and M. New virions are formed by a process of budding through the cell membrane (Brown & Crick, 1980).

The nucleotide sequence of the mRNA encoding rabies virus glycoprotein was first reported in 1981 (Anilionis et al., 1981). All structural protein genes, except that coding for the L protein have since been cloned (WHO, 1984) and the glycoprotein gene has been successfully inserted into *Escherichia coli* (Yelverton et al., 1983; Lathe et al., 1984), *Saccharomyces cerevisiae* (Lathe et al., 1984), vaccinia virus (Kieny et al., 1985), and simian virus 40, adenovirus, and bovine papilloma virus for expression in mammalian cells (Kieny et al., 1985).

4. **Rabies serotypes and related viruses**

Members of the *Lyssavirus* genus contain a common cross-reacting N protein (Brown et al., 1979) which can be detected by standard fluorescent antibody, complement fixation, and precipitation techniques (WHO, 1980c). They are distinguished by virus-neutralisation and cross-protection tests, indicating that their surface glycoproteins are different (Shope, 1975). The application of monoclonal antibody technology to the study of antigenic relationships among rhabdoviruses (Wiktor & Koprowski, 1978) has confirmed the distinction between rabies and rabies-related viruses and has revealed extensive antigenic variation in both glycoprotein and nucleocapsid proteins of a number of laboratory and street
strains (Flamand et al, 1980a; Flamand et al, 1980b; Wiktor et al, 1980; Schneider et al, 1985). Analysis of several hundred isolates from animals and man in different parts of the world has revealed both geographic and species patterns of reactivity (Wiktor et al, 1980; Koprowski et al, 1985; Schneider et al, 1985). Similarly, live virus strains used for the immunization of animals can be distinguished from wild-type viruses. When the antigenic makeup of a large number of field isolates were compared with that of the PM-HDCS vaccine strain, the percentage of common antigenic determinants ranged from 44 to 100 per cent (Wiktor, 1985). The relevance of these observations to recommendations concerning strains for vaccine and antiserum production has yet to be established, although preliminary investigations provide no indication that antigenic differences account for vaccination failures.

5. Effects of physical and chemical agents
Rabies virus is inactivated below pH 4 and above pH 10 (Turner & Kaplan, 1967). It has a half-life of about 4 hours at 40° C and is inactivated within ten minutes at 60° C, but it is stable for several days at 0-4° C and for several years when frozen at -70° C, or lyophilised and held at 0-4° C (Turner & Kaplan, 1967; WHO, 1980c). The virus is destroyed by quaternary ammonium disinfectants (e.g., benzalkonium and cetrimonium at dilutions of 1/1000), 1 per cent soap solutions, ionic and non-ionic detergents, 5 per cent iodine, most common organic solvents (45 per cent alcohol, ether and chloroform), formalin, β-propiolactone, acetyleneimine, tri(n-butyl)phosphate, proteolytic enzymes, UV light and ionizing irradiation. (Lubinski & Prausnitz-Breslau, 1926; Kaplan et al, 1966; Turner & Kaplan, 1967; Neurath et al, 1972; Wiktor et al, 1972a; Habel, 1973). An important component of postexposure treatment is the thorough cleansing of the wound with soap or detergent.
CHAPTER 4.
TRANSMISSION AND PATHOGENESIS.

1. Transmission

By far the most common mode of infection in man is by the bite of a rabid animal or the contamination of scratch wounds by virus-laden saliva. Intact skin appears impenetrable to the virus, but infection across undamaged mucous membranes of the mouth, conjunctiva, anus and genitalia, either directly from rabid animals (Mantell, 1833; Babes, 1912; Lubinski & Prausnitz-Breslau, 1926; Leach & Johnson, 1940), or indirectly through licking contaminated rope or material (Mantell, 1833) are feasible. Infection by aerosol transmission has also been amply demonstrated in laboratory animals (Constantine, 1962; Atanasiu, 1965; Hronovsky' & Benda, 1969a and b), and has been implicated in human infection acquired in bat-infested caverns in Texas and in several laboratory accidents (Irons et al 1957; Humphrey et al 1960; Winkler et al 1973; MMWR, 1977). "Rage de laboratoire", the consequence of receiving incompletely inactivated fixed rabies virus in human vaccines, is well documented in the older literature (Remlinger, 1935) and an outbreak involving 18 persons occurred in Brazil in 1960 (Para, 1965).

Man to man transmission by transplantation of infected corneas has been reported in five instances with the diagnosis in the donors only being made retrospectively (Houff et al, 1979; MMWR, 1980; MMWR, 1981a; Sureau et al 1981). It has also reportedly occurred in an incident involving several young boys in the North-West Frontier Province of Pakistan who were infected by saliva applied to circumcision wounds by a surgeon-barber with early rabies. Occasional cases of human-to-human transmission are described in the older literature, and in a large
Indian survey 1 of 11,134 contacts of human cases developed the disease (Greenwood 1945). Judging by the absence of reports of cases in nursing and medical staff, it seems to be a rare event. None the less, medical attendants are at genuine risk of being bitten or contaminated by saliva, (Meyer 1957; Anderson et al 1966; Emmons et al 1973; MMWR 1977b; Lintjorn 1982), and especially to aerosols generated by airway care, and should be considered for postexposure therapy. Kissing and coitus as routes of human to human transmission have also been described in the older literature (Mantell 1833; Lubinski & Prausnitz-Breslau 1926).

Vertical transmission after natural infection is recorded in various species (Konradi 1908; Martel et al 1973; Afshar 1979; Howard 1981) but not in man, although there are instances of women dying from rabies shortly after parturition (Jellesic 1958; Bisseru 1972; Martel et al 1973; Spence et al 1974-75). Oral transmission through cannibalism occurs in laboratory animals (Fischmann & Ward 1968; Nicholson & Bauer 1981) but has never been described in man; its role in nature is unknown. Reference to infection in weanling animals possibly through the consumption of infected milk was made in a recent review (Afshar 1979).

Although many patients can relate the circumstances of their exposure, recent experience in the USA indicates that perhaps 50% are unable to do so (MMWR 1981b).

2. Pathogenesis

In 1769 Morgagni noted that parasthesiae at the bite site often heralded the onset of rabies and he postulated that neural rather than haematogenous spread of virus had occurred (Wright, 1959). There is now overwhelming evidence in support of neural translocation whereas the dissemination of virus in the blood stream is unsupported by critical evaluation. As outlined below, the pathogenesis of rabies involves the following stages: entry of virus into peripheral nerves; centripetal movement of
virus to the cord and brain; viral replication within the CNS; centrifugal movement of virus back along nerve routes to salivary glands and other tissues where viral replication and release occur. The survival of the virus in nature is wholly dependent upon the behavioural changes which rabies encephalitis causes.

**Entry of virus into peripheral nerves:** Recent reports suggest that the rabies virus receptors coincide with the distribution of acetylcholine receptors (Watson et al 1981; Lentz et al, 1982, Lentz et al, 1983). However, rabies virus can enter cells independently of the nicotinic acetylcholine receptor complex (Reagan et al, 1985). Using sequential tissue titrations, immunofluorescence, and electron microscopy, it has been shown that virus localises initially at motor end-plates near the site of inoculation, infects and replicates in myocytes, is shed into extracellular spaces and involves neuromuscular and neurotendinal spindles to enter peripheral nerves (Murphy et al 1973a, 1973b, 1974; Murphy, 1977; Watson et al, 1981). Only during this early stage does the virus seem susceptible to postexposure therapy.

**Translocation to the CNS:** In 1889 Di Vestea & Zagari inoculated rabies virus into sciatic and median nerves and showed that virus was recovered first in the appropriate areas of the spinal cord and that its translocation could be interrupted by section of the cord. In 1925 Goodpasture noted that cytopathic changes in the ganglia and CNS corresponded anatomically to the sites of virus inoculation, again inferring neural transmission. Further studies gave support to this hypothesis. Thus, initial CNS infection, as shown by virus isolation (Webster 1937, Kligler & Bernkopf 1943; Huygelman & Mortelmans, 1959; Baer et al 1965, 1968; Johnson 1965; Otani 1965; Petrovic & Timm, 1969), and immunofluorescence (Johnson 1965; Otani 1965; Yamamoto et al, 1965), occurs in the lumbar cord following hind-limb inoculation, the upper cord following fore-limb inoculation, and the brainstem following masseter inoculation. Rabies virus moves from peripheral sites to the CNS at a rate crudely estimated at 3mm/h (Dean et al, 1963a).
Transition is unaffected by removal of the perineurium, epineurium or perineural epithelium (Baer et al 1965, 1968), but is blocked by local anaesthetics (Kaplan et al 1962, Dean et al, 1963b), colchicine and vinblastine (Bijinga & Heaney, 1978; Tsiang, 1979). No replication occurs within the axoplasm, which is devoid of the necessary organelles. Amplification may occur, however, within neurones of the dorsal route ganglia (Yamamoto et al, 1965; Murphy et al, 1973a).

CNS involvement: From the cord, ascent to the brain is rapid with initial distribution influenced by the site of virus inoculation (Murphy, 1977). CSF is of doubtful importance in viral dissemination since in man it is rarely infectious ante-mortem (Kent & Finegold, 1960; Duenas et al, 1973; Bhatt et al, 1974; Cohen et al, 1976; Reis et al, 1976). There is extensive viral replication in the brain, which probably supports several growth cycles before onset of clinical effects (Johnson, 1965; Schneider, 1969). Terminally, there is widespread CNS involvement in man and animals (Leach & Johnson, 1940; Dupont & Earle, 1965; Murphy, 1977), but few neurones infected with street virus show structural abnormalities (Miyamoto & Matsumoto, 1967). The nature of the profound neural disorder is thus obscure though, according to one hypothesis, the greater localisation of virus to the limbic system provides a clinicopathologic correlate for the abnormal behaviour and aggression necessary for virus perpetuation (Johnson, 1971).

Interestingly, recent work suggests that opiate (Münzel & Koschel, 1981) and acetylcholine (Tsiang, 1982) receptors of infected cells are impaired, implying disordered neuro-transmission as the cause for the progressive neural dysfunction.

Movement From The CNS: Following invasion of the CNS, "street" and occasionally "fixed" strains of rabies virus move along axoplasmic routes to salivary glands and other tissues (Bertarelli, 1904; Dean et al, 1963a; Fischmann 1969; Garcia-Tomayo et al, 1972; Murphy et al, 1973b). Many sites may be involved including
lacrimal glands, myocardium, skeletal muscle, lung, liver, adrenal glands, the retina, cornea, taste buds, sebaceous glands and hair follicles, particularly those of the head and neck (Leach & Johnson, 1940; Beran et al, 1972; Smith et al, 1972; Duenas et al, 1973; Reis et al, 1976; Houff et al, 1979). Sites with short neural connections to the CNS are usually the first to be infected and immunofluorescence of corneal impressions or skin biopsies may establish the diagnosis antemortem (Cifuentes et al, 1971; Bryceson et al, 1975).

The most important site for virus transmission is the salivary gland mucosal epithelium which represents the major source of virus shed into secretions (Dierks et al, 1969; Constantine et al, 1972; Dierks, 1975). Occasionally the titres of street virus in salivary gland exceed those of brain indicating efficient replication in mucous acinar cells (Vaughn et al, 1963, & 1965; Parker & Wilsnack, 1966; Dierks et al, 1969). Other commonly infected sites which may shed virus into oronasal secretions are the olfactory end-organs in the nares and the neuroepithelium of taste-buds (Constantine et al, 1972; Murphy et al, 1973a; Micholson & Bauer, 1981). Experimentally, about 48 per cent of dogs (Vaughn et al, 1965), 88 per cent of cats (Vaughn et al, 1963), 42 per cent of foxes (Sikes, 1962), and 83 per cent of skunks (Sikes, 1962) excrete virus in saliva, though gland involvement of naturally-infected animals may reach 90 per cent (Schaaf & Schaal, 1968). The behaviour and appearance of animals are poor guides to their infectivity as virus shedding may precede illness by days (Baer & Bales, 1967; Vaughn et al, 1963 & 1965; Wilsnack, 1966). Moreover, there are well documented instances of apparently healthy dogs excreting virus intermittently over periods of many months (Veeraraghavan, 1970; Fekadu et al, 1981) and of apparently healthy animals outliving their victims (Veeraraghavan, 1966).
1. Factors influencing infection

Bites inflicted by rabid animals do not necessarily cause disease. In man, mortality rates of 35-71 per cent have been recorded when the infectivity of the biting animal is established by the death of another person or animal (Semple, 1919; Cornwall, 1923; Nikolic, 1952; Gremliza, 1953; Veeraraghavan, 1969). Observations made in the era before vaccination indicate considerable variation in human mortality after dog and cat bites, ranging from 2.5 to 44% (Babes, 1912). Apart from species differences in the susceptibility to the virus, several factors modify the outcome. First, the infectivity of the saliva may vary (Sikes, 1962; Vaughn et al, 1963; Vaughn et al, 1965). Secondly, the site and severity (deep vs superficial, and multiple vs single) of the bites. Severe head and neck bites carry the greatest risk, followed by bites to the upper limb, (particularly the richly innervated hand) then the lower limb and trunk (Babes, 1912; van Rooyen & Rhodes, 1948; Gremliza, 1953; Shah & Jaswal, 1976). However, the location and severity of wounds are influenced by the species of biting animal which in turn affects the amount of saliva deposited in the wound and, probably, its infectivity. Finally, the intervention of clothing reduces mortality by 60-80% compared with wounds inflicted through bare skin (McKendrick, 1940).

2. Incubation period

The incubation period is highly variable, ranging from seven days (Chopra et al, 1980) to several or more years, but in most cases it ranges from 30 to 90 days (Ahuja & Brooks, 1950; Wang, 1956; Gavrilov et al, 1961, 1967; Suri & Chugh, 1975; Anderson et al, 1984). Exceptionally, in the case of ‘rage de laboratoire’, the
The incubation period can be as short as 4 days (Para, 1965). In mice, the greater the infecting dose the shorter the incubation period (Nikolitsch, 1958); similarly in man, an association between the incubation period and severity of exposure has been noted (Held et al, 1967). The shortest incubations are seen with multiple severe wounds, especially those involving the head (Semple, 1919; Blatt et al, 1938; Ahuja & Brooks, 1950; Wang, 1956; Suri & Chugh, 1975). Children tend to have shorter incubation periods than adults (Babes, 1912; Ahuja & Brooks, 1950; Wang, 1956), though this may in part be due to them being bitten more severely and frequently to the face. Wolves often inflict multiple deep wounds and rabies following wolf-bites tend to have a short incubation period. Incubation periods are also significantly shorter in patients with 'failed' postexposure treatment (Wang, 1956; Held et al, 1967; Suri & Chugh, 1975). Until recently, the only explanation offered was that treatment was relatively more efficient at preventing disease with long rather than short incubation. An alternative hypothesis, which is supported by experimental data, is that an immuno-pathogenic response may actually accelerate the disease process, causing the 'early death phenomenon'. This is observed in vaccinated animals with low levels of antibody, who die more rapidly when challenged with rabies virus than unvaccinated control animals without antibody (Sikes et al, 1971a; Blancou et al, 1980; Andral & Blancou, 1981; Prabhakar & Nathanson, 1981; Smith et al, 1982).

Unless secondary infection occurs, any wounds inflicted heal uneventfully during the incubation period. Exactly what happens to the virus throughout this stage is uncertain, although in a mouse model of infection, amputation experiments suggest that the virus may remain at or near the wound site for a prolonged period (Baer & Cleary, 1972).

3. The prodrome

The onset of human rabies is marked by 2 to 7 days of prodromal symptoms that are
almost entirely non-specific comprising fever, malaise, anorexia, nausea, vomiting, diarrhoea, sore throat, cough, myalgia and headache as the most common complaints (Blatt et al, 1938; Dupont & Earle, 1965; Anderson et al, 1984). Behaviour disturbances are often noted, including anxiety, depression, stupor, hyperactivity, aggression, delirium and intolerance to tactile, auditory and visual stimuli (Babes, 1912; Leach & Johnson, 1940; Cifuentes et al, 1971; Emmons et al, 1973; Bhatt et al, 1974; Sung et al, 1976; Conomy et al, 1977; De Wet, 1980; Lintjorn, 1982).

The patient may complain of insomnia, nightmares (Babes, 1912; Lubinski & Prausnitz, 1926), hallucinations (Gamaleia, 1887; Babes, 1912; Gonzalez-Ungulo, 1970; MMWR, 1971; Warrell et al, 1976; Escobar-Izquierdo et al, 1980), and rarely priapism (Hunter, 1793; Gamaleia, 1887; Babes, 1912; Talaulicar, 1977; De Wet, 1980). An early symptom of diagnostic significance is of abnormal sensation involving the bitten area, most commonly pain or paraesthesiae noted in about 44 per cent of cases (Dupont & Earle, 1965; Suri & Chugh, 1975; Wilson et al, 1975; Warrell, 1976; Anderson et al, 1984). Few objective signs appear early in the clinical course, so unless the doctor's attention is drawn to a recent exposure or healed bite wounds are noticed it is unlikely that the diagnosis will be considered.

4. Patterns of disease

The prodrome is followed by one of two basic clinical patterns: the more common 'furious' form characterised by hyperexcitability, spasms and hydrophobia; or 'dumb' rabies featuring an ascending paralysis. For descriptive purposes they are considered separately, although 'furious' rabies is often accompanied by paralysis and 'dumb' rabies may herald spasms and hydrophobia. Rabies should therefore be regarded as having a broad clinical spectrum rather than two distinct forms.

The onset of furious rabies is marked by increasing insomnia and periods of extreme agitation, delirium, hyperactivity and purposeless movements, either occurring spontaneously or provoked by any tactile, auditory, visual, or olfactory
stimuli. Often these episodes are accompanied by frothing at the mouth, difficulty in swallowing and intense spasms affecting the muscles of deglutition and accessory muscles of respiration and lasting for 5-15 seconds. They are followed minutes later by lucid intervals with the patient lying anxious and exhausted in bed (Blatt et al, 1938; Hattwick, 1974; California Morbidity, 1979). The spasms characteristically follow attempts to drink but may be precipitated by other stimuli, e.g., eating, swallowing accumulated saliva, or by a draught of air (aerophobia) (Blatt et al, 1938; Wilson et al, 1975; Warrell et al, 1976; Anderson et al, 1984). Many patients look intensely frightened, the appearance of fear being heightened by a proptotic stare, dilated pupils and an open mouth (Blatt et al, 1938; Sung et al, 1976; Newman & Grace, 1978). Often the respirations are shallow and rapid and there may be chest pain and tightness (Humphrey et al, 1960; Kent & Finegold, 1960: Wilson et al, 1975).

Approximately 50 per cent of cases exhibit hydrophobia, an overwhelming fear of water precipitated by attempts to drink, and less frequently, by the sight, sound or mention of water or other fluids (Dupont & Earle, 1965; Suri & Chugh, 1975; Wilson et al, 1975; Anderson et al, 1984). Hydrophobia typically starts with uncontrolled jerking movements of the hand, arm or body as fluid is brought to the mouth; the head jolts backward, the arms upward, fluid is spilled and spasms are induced. Attempts to swallow are defeated by coughing, retching, vomiting, aspiration, opisthotonous, asphyxiation and convulsions which end in death in 28% of cases (Dupont & Earle, 1965). Often the patient struggles violently, he may attack attendants or bolt from the room.

The excitement phase sees an initial increase then decrease in the frequency and severity of spasms. The patient is usually febrile and may develop a variety of abnormalities, including nuchal rigidity, photophobia, fasciculations and paresis, particularly at the site of exposure, cerebellar signs, cranial nerve palsies, hypoor hyper-reflexia, extensor plantar responses, focal or generalised convulsions and a variety of autonomic disturbances (Anonymous, BMJ, 1975). Deterioration is
marked by the evolution of a flaccid paralysis and onset of coma, by irregular patterns of respiration and by potentially fatal complications. Untreated cases survive for an average of 3-7 days once symptoms develop (Leach & Johnson, 1940; Gremliza, 1953; Suri & Chugh, 1975; Warrell et al, 1976).

Dumb rabies is far more likely to pose diagnostic problems since the pharyngeal spasms and hydrophobia so characteristic of rabies hardly ever appear. Paralytic rabies has an incidence of 14-60 per cent in reported series of cases, there being approximately 4 furious cases for every dumb one overall (Babes, 1912; Knutti, 1929; Hurst & Pawan 1968; Leach & Johnson, 1940; Anderson et al, 1984). The incidence is probably higher since the onset of a flaccid paralysis some months after a minor or forgotten exposure is likely to be diagnosed as polio, transverse myelitis, or Guillain-Barre’s syndrome. To stress this point, it should be noted that at least 5 corneas have unwittingly been transplanted from persons with paralytic rabies.

'Dumb' or 'paralytic' rabies normally begins with typical prodromal symptoms, occasionally with hyperaesthesiae or pain localised to the bite-site (Gamaleia, 1887). Anaesthesia may be present (Knutti, 1929), but is seldom a dominant feature (Love, 1944). The paralysis often involves the bitten limb initially then spreads rapidly and symmetrically. Sphincter control is typically lost and paralysis of the muscles of deglutition, articulation and respiration normally occur as a terminal event. The course can be modified at any stage by the appearance of spasms, hydrophobia and convulsions. Survival tends to be longer than for patients with furious rabies, the average period from several series being 7-12 days (Gamaleia, 1887; Love, 1944; Chopra et al, 1980).

5. Complications
Complications involving the cardiovascular, respiratory and neurological systems become especially prominent when the course is prolonged by intensive therapy,
although disturbances affecting other systems may also aggravate the clinical course.

As a sequel to inflammatory or hypoxic cerebral oedema, raised intracranial pressure contributes to the decreased level of consciousness and to focal and generalised convulsions. Other CNS complications include disturbances of thermoregulation, diabetes insipidus, autonomic dysfunction affecting fluid and electrolyte balance and blood pressure (Bhatt et al, 1974; Lopez et al, 1975; Cohen et al, 1976; Gode et al, 1976; Maton et al, 1976), convulsions in two-thirds of cases (Blatt et al, 1938), and irregular breathing patterns consistent with lesions at the mid-pontine level or ponto-medullary junction (Warrell et al, 1976).

The clinical course is almost invariably complicated by cardiac dysrhythmias of virtually any kind (Ross & Armentrout, 1962; Bhatt et al, 1974; Cohen et al, 1976; Gode et al, 1976; Maton et al, 1976; Warrell et al, 1976). Several investigators have noted histological evidence of a myocarditis (Ross & Armentrout, 1962; Cheetham et al, 1970; Warrell et al, 1976); ECG changes supporting the diagnosis of myocarditis have also been found (Ross & Armentrout, 1962) and, when searched for, virus has been isolated from myocardium in 52% of cases (Ross & Armentrout, 1962; Cifuentes et al, 1971; Duenas et al, 1973). Severe hypotension with pulmonary oedema (Cheetham et al, 1970; Garcia-Tomayo et al, 1972; Gode et al, 1976), congestive cardiac failure (Bhatt et al, 1974) or acute renal failure (MMWR, 1981c) is a common finding in the later stages of the disease (Lopez et al, 1975; Cohen et al, 1976; Maton et al, 1976; Grani et al, 1978; Cundy, 1980) and may be partly or wholly caused by myocarditis, hypoxia, autonomic dysfunction or hypovolaemia.

Respiratory disturbances occur in all cases. Blood-gas and pH abnormalities complicate the early clinical course partly because of hyperventilation (Bhatt et al, 1974; Wilson et al, 1975). Thereafter death is often precipitated by asphyxiation caused by hydrophobic spasms, convulsions or bulbar and respiratory paralysis. Certain complications, notably bronchopneumonia and collapse and consolidation,
invariably occur after tracheostomy and artificial ventilation and are potentiated by increased secretions, heart failure and aspiration (Bhatt et al, 1974; Lopez et al, 1975; Gode et al, 1976). These together with the cerebral effects on ventilation significantly lower the arterial pO2, which is often found as a terminal event (Warrell et al, 1976).

Hypovolaemia, electrolyte imbalance, and pH disturbances, arising from fluid deprivation, inappropriate secretion of anti-diuretic hormone, diabetes insipidus, excessive secretions, ileus, and ventilation-perfusion defects, frequently complicate the late clinical course. Some gastrointestinal complications are occasionally seen including gastro-oesophageal tears (Kent & Finegold, 1960; Rodrigues et al, 1974), gastritis (Lopez et al, 1975), frank ulceration (Hattwick et al, 1972b) haematemesis (Blatt et al, 1938; Cifuentes et al, 1971; Cohen et al, 1976), pancreatitis (Bhatt et al, 1974) and ileus (Bhatt et al, 1974; Gode et al, 1976).

6. Differential Diagnosis

With such non-specific prodromal features, rabies is unlikely to be considered during the early stages unless a history of bite-exposure is volunteered or obvious spasms or hydrophobia are present. Recent American experience showed rabies to be considered for only 3 of 21 patients on first visit to a physician and for only 7 of 23 on admission to hospital (Anderson et al, 1984). The admission diagnoses included viral encephalitis, polio, post-infectious encephalitis, vaccine reaction, Guillain-Barre syndrome, brain abscess, cerebrovascular accident, brain tumour, tetanus, phenothiazine toxicity, psychosis, rabies phobia, pneumonia, myocardial infarction, dissecting aortic aneurysm and arteritis. Previous reviews mention 'rage de laboratoire', malingering, delirium tremens, botulism, and poisoning by strychnine and other plant derivatives in the differential diagnosis (Babes, 1912; Para, 1965; Bisseru, 1972; Shope, 1976; Warrell, 1976).

Important distinguishing features for some of the above are outlined over:
Rabies Phobia: Most patients with rabies phobia or hysteria are afebrile and prodromal symptoms are absent. They characteristically refuse to drink; if they do drink, they exhibit no jerkiness of hand, arm or head, and no convincing inspiratory spasms (Semple, 1919; Ahuja & Brooks, 1950; Bisseru, 1972; Lintjørn, 1982). Fanning a patient with genuine rabies often invokes inspiratory spasms (Wilson et al, 1975) - a feature not seen with rabies phobia.

Tetanus: The incubation period is shorter than for rabies although in many instances it cannot be determined. The presence of trismus and persistence of increased muscular tone between spasms in tetanus normally differentiate it from rabies. The results of lumbar puncture are also helpful since they are abnormal in 60-90% of cases of rabies (Anderson et al, 1984) but rarely so in tetanus (Adams et al, 1969; Brain & Walton, 1969).

Polio: Unlike rabies, the fever of polio usually abates with the evolution of paralysis; sensory disturbances, spasms and hydrophobia are also absent.

Vaccine Reactions: Neuroparalytic reactions are usually seen 8-21 days after the onset of vaccination (Horack, 1939; Redewill & Underwood, 1947; Sellers, 1947; Pait & Pearson 1949; Appelbaum et al, 1953), well within the incubation period of rabies. Spasms and hydrophobia are absent and recovery is the rule. There is also a notable absence of virus and Negri bodies in the brains of those who die.

Guillain-Barre' Syndrome: This cannot be distinguished on clinical grounds or by examination of the CSF during the early stages. Recovery from Guillain-Barre' syndrome is generally the rule, however, and consciousness is fully retained. Rabies should always be considered if there is any deviation from the normal clinical course.
Viral Encephalitis: Hydrophobia and spasms are absent in encephalitis and are seen in only one-half of cases with rabies. Since rabies is otherwise indistinguishable from other encephalitides it should always be considered for patients coming from endemic areas.

Poisoning and Drugs: Strychnine poisoning may cause paroxysmal spasms of respiratory muscles resembling those of rabies, but there are no prodromal symptoms and no fever. Similarly, the dystonia and oculogyric crises of phenothiazine toxicity are unlikely to be confused with the spasms of rabies.

7. Diagnostic Testing

Non-specific examinations: The blood white cell count is often elevated (10-20 000/mm³) with a polymorph predominance; occasionally it is normal or in excess of 30 000 (Ross & Armentrout, 1962; Dehner, 1970; Bhatt et al, 1974; Rodrigues et al, 1974; MMWR, 1981b; Prakash et al, 1981). CSF examination typically shows a normal opening pressure and a mixed pleocytosis rarely in excess of 300/mm³ in 60-90 per cent of cases (Anderson et al, 1984). The CSF protein is modestly elevated in one-quarter of cases during the first week of illness. Later it is raised to an average concentration of about 100 mg/dL in 80 per cent of cases (Anderson et al, 1984). CAT brain scans are reportedly normal, and EEGs usually show diffuse slow-wave activity or an iso-electric recording (Bhatt et al, 1974; Cohen et al, 1976; Maton et al, 1976; Houff et al, 1976).

Intra-vitam Diagnosis: Laboratory confirmation of the diagnosis is possible before death by finding specific fluorescence in corneal impressions (obtained by gently abrading the cornea with a microscope slide) (Cifuentes et al, 1971; Larghi et al, 1973; Koch et al, 1975), skin biopsy (normally taken from the neck or face to show viral antigen in sensory nerve endings) (Smith et al, 1972; Bryceson et al, 1975;
Blenden, 1978), or brain biopsy (rarely indicated) (Emmons et al, 1973); by recovery of rabies virus from saliva, throat swab, tracheal aspirates (Duffy et al, 1974; Bhatt et al, 1974; Cohen et al, 1976; Reis et al, 1976; MMWR, 1981c) and exceptionally from tears (Bhatt et al, 1974), urine sediment (Bhatt et al, 1974) and CSF (Bhatt et al, 1974); and finally by detecting rabies antibody in serum (Bhatt et al, 1974; Reis et al, 1976; Anderson et al, 1984) or CSF (Hattwick et al, 1972a; Porras et al, 1976; Schuller et al, 1979). Virus can often be recovered human saliva, throat swabs or tracheal aspirates during the first two weeks of illness, but later attempts at virus isolation or immunofluorescence are often negative (Anderson et al, 1984), presumably because the virus has been 'neutralized' by antibody. CSF rarely contains infectious virus antemortem (Kent & Finegold, 1960; Duenas et al, 1973; Bhatt et al, 1974; Cohen et al, 1976; Reis et al, 1976), although at autopsy it can occasionally be found (Kent & Finegold, 1960; Duenas et al, 1973). There are no adequately documented cases of viraemia, and attempts to isolate the virus from the blood of human cases have been unsuccessful (Semple, 1919; Leach & Johnson, 1940; Duenas et al, 1973; Bhatt et al, 1974; Duffy et al, 1974).

How useful are these methods clinically? Recent American experience showed the detection of serum antibody in unvaccinated subjects to be the most successful overall, with a cumulative detection rate of 50 per cent by days 5-8, 67 per cent by days 9-12, and 100 per cent by days 13-16 (Anderson et al, 1984). Antibody appeared much later in CSF than in blood and was of no help in early diagnosis. As might be expected, antibody detection and virus isolation were inversely related, with virus recovery from 60 per cent on days 0-4, about 32 per cent on days 5-8 and 9-12, and 18 per cent on days 13-16. Similarly, study of seven South American cases showed virus shedding from 100 per cent on days 0-4, 50 per cent on days 5-8, 33 per cent on days 9-12, and from no-one on days 13-16 (Reis et al, 1976). Virus isolation is a time-consuming procedure and it is generally several weeks into the clinical illness before the diagnosis is
established or refuted by either method.

When positive, immunofluorescence tests establish the diagnosis rapidly. In the American series, immunofluorescent staining for rabies antigen in corneal impressions or neck skin biopsy specimens was diagnostic in only about 50 per cent of cases early in the clinical course, and occasionally the corneal impression test gave false-positive results (Anderson et al, 1984). In the South American study, daily examination of corneal impressions gave negative results in all 7 cases (Reis et al, 1976).

Postmortem Diagnosis: The postmortem diagnosis of rabies in both animals and man for many years depended on the demonstration of Negri bodies in the brain (Negri, 1903), which appear as intracytoplasmic, eosinophilic inclusions generally measuring 0.2-27 μm in diameter (Tierkel, 1973), but may be absent in as many as 30% of cases (Dupont & Earle, 1965). They are usually found in more or less undamaged nerve cells, particularly in Ammon's horn, cerebral cortex, medulla, and cerebellum. After staining with Seller's methylene blue basic fuchsin, they show a heterogeneous magenta-red matrix containing dark-blue-to-black inclusions known as "Innerkörperchen" (Tierkel, 1973). Structures lacking this inner basophilic core but otherwise similar to Negri bodies are known as "Lyssa" bodies (Goodpasture, 1925). Both structures contain rabies antigen and are found in great numbers in rabid brain (Miyamoto & Matsumoto, 1965; Sung et al, 1976), but neither is pathognomic for rabies (Derakhshan, 1975; Derakhshan et al, 1978).

Intracerebral mouse inoculation with a homogenate of brain coupled with immunofluorescence or the microscopic examination of mouse brain tissue for Negri bodies is an extremely valuable confirmatory test (Leach & Johnson, 1940; Cifuentes et al, 1971; Reis et al, 1976), although if the patient's survival is prolonged until after the appearance of antibody, attempts at virus isolation almost invariably yield negative results (Rubin et al, 1970; Hattwick et al, 1972; Emmons
et al, 1973; Maton et al, 1976; Grâni et al, 1978). Demonstrating rabies antigen in sections of human brain by immunofluorescence (IF) is the most rapid specific test (Goldwasser & Kissling, 1958; Jentzsch, 1967), but again it is often negative in cases with prolonged survival and requires the material for examination to be in a good non-putrefied state (Wachendörfer, 1966). However, IF can still be carried out if the specimen has been fixed in formalin (Umoh & Blenden, 1981). In addition to nervous tissue, rabies virus can be recovered in a variable proportion of cases from the lacrimal and salivary glands, myocardium, skeletal muscle, lung, liver, kidney, peripheral nerves and adrenal glands (Leach & Johnson, 1940; Beran et al, 1972; Duenas et al, 1973; Reis et al, 1976; Houff et al, 1979). Finally, electron-microscopy can establish the diagnosis by revealing the presence of typical bullet-shaped virus particles in postmortem brain (Baer et al, 1982).

8. Pathology and management

In contrast to most viral encephalitides there is a relative lack of cytolysis and inflammation. The changes noted are perivascular cuffing, neuronophagia and neural degeneration, and proliferation of the capsular cells surrounding ganglionic neurones and Negri bodies (Perl, 1975). This inevitably fired speculation that intensive supportive care might eventually lead to recovery. The survival of two patients with proven rabies for periods of 64 and 133 days (Rubin et al, 1970; Emmons et al, 1973), together with the recovery of 3 patients with probable rabies (Hattwick et al, 1972; Porras et al, 1976; MMWR,1977a), has further raised hopes although one survivor was left with severe neurological impairment and all 3 were vaccinated prior to the onset of symptoms. Life can undoubtedly be prolonged by meticulous supportive care, although it is equally clear from recent experience that it is the extent and severity of the encephalitis that are major barriers to survival (Bhatt et al, 1974; Lopez et al, 1975; Cohen et al, 1976; Gode et al, 1976; Maton et al, 1976; MMWR, 1981b).
Many patients have been given the benefit of treatment with antiviral agents or immunotherapy, but the measures tried have all been singularly unhelpful. Steroids impair antibody production (Burns et al, 1960) and depress immunity in laboratory animals (Soave, 1962) and have been used without success in man (Hattwick, 1972b; Cohen et al, 1976; Gräni et al, 1978) and are generally contraindicated. No benefit has been gained from cytosine arabinoside (Hattwick et al, 1972b; Gode et al, 1976) or administration of rabies antibody either by the intrathecal (Emmons et al, 1973; Hattwick et al, 1976) or intramuscular routes (Lopez et al, 1975; Cohen et al, 1976; Maton et al, 1976; Gräni et al, 1978; MMWR, 1981c), or from transfusion of a unit of immune whole blood (Hattwick et al, 1972b). In fact, the discovery of 'lytic' antibody (Wiktor et al, 1968) led to the view that passively administered antibody after onset of disease might actually be harmful. Exchange transfusion was therefore tried; this too was unhelpful (Cifuentes et al, 1971). Similar lack of success was noted with Freund's adjuvant (Gode et al, 1976) and a polyanion interferon inducer (Cifuentes et al, 1971; Lopez et al, 1975). Interferon itself has been tried in several patients by intramuscular and intrathecal routes; none survived and interestingly the virus could still be isolated from one patient five days after onset of treatment (Gräni et al, 1978; MMWR, 1981c).
CHAPTER 6.
PRE- AND POST-EXPOSURE TREATMENT AND ASSESSMENT
OF IMMUNE STATUS.

1. Wound treatment
The essential components of postexposure prophylaxis are the local treatment of wounds and active and passive immunisation. The local application of interferon and interferon inducers have yet to be tried in man though they are of proven value in laboratory animals (Nemes et al, 1969; Harmon & Janis, 1975; Baer et al, 1977; Baer et al, 1979). Experimentally, the incidence of rabies can be markedly reduced by local therapy alone. It is of maximal value when applied promptly after exposure but should not be neglected even if several hours or days have elapsed (WHO, 1984).
Recommended first-aid procedures are the thorough cleansing of the wound with copious amounts of soap and water, detergent, or water alone sufficient to make the wound bleed; the application of a virucidal substance, either 40-70% alcohol (Cohen et al, 1962; Kaplan et al, 1966), tincture or aqueous solutions of iodine (Kaplan et al, 1966), or a quaternary ammonium compound (QAC) eg 1-2% benzalkonium chloride (Shaughnessy & Zichis, 1943, 1954; Kaplan et al, 1962), 0.1% cetrimonium bromide (Cetavlon)(Kaplan et al, 1966); passive immunisation; and antitetanus procedures and antibiotics when indicated. QAC may be neutralised by tapwater in "hardwater" areas as well as by soap; wounds cleansed with a soap solution should therefore be thoroughly rinsed with water before application of a QAC (WHO, 1984). Bite wounds should not be immediately sutured (Perez Gallardo et al, 1958); if suturing is necessary then antiserum or immune globulin should be infiltrated locally.
2. Neurotissue vaccines prepared from adult brain

With the increasing demand for Pasteur's treatment it soon became evident that difficulties in maintaining large numbers of freshly infected cords would restrict its application. This was resolved by the introduction of glycerol as a vaccine preservative (Roux, 1887; Calmette, 1891). Other modifications were soon advocated. Höyges reduced virulence by diluting infected rabbit cords 1 in 10 to 1 in 5,000 in NaCl (Anon, 1887); heat inactivation was introduced by Puscariu & Vesesco (1895) (at temperatures of 30°C to 80°C for 10 minutes) and Babes (1912) (exposure at 58°C for different times) to overcome the vagaries of dessication; and Fermi (1908) first used a chemical method, 1% phenol, to attenuate the virus. Fermi's method differed from those previously used in so far that there was no gradation of virulence of successive doses; this greatly simplified the manufacturing process and the method became widely accepted. Concern about the danger of inoculating residual live virus led Semple to modify the phenol treatment so as to render the vaccine non-infectious but still to retain its immunizing capacity (Semple, 1911). Semple's method became well established and although other methods of inactivation were introduced, namely - ether (Alvisatos, 1922; Hempt, 1925), chloroform (Kelser, 1930), ether and phenol (Hempt, 1938), formalin (Otten, 1947) and ultraviolet light (Webster & Casals, 1942), they never became so universally accepted. Today the infected brain tissue of adult rabbits, sheep and goats remains the principal source of virus for vaccine production in many parts of the world and Semple vaccine is the most widely used. There have been several minor modifications in its preparation; complete inactivation of the virus is usually achieved with phenol at a concentration of 0.5 to 1 per cent, at temperatures between 20 and 30°C for 48 to 72 hours (Turner, 1969). Due to poor antigenicity, courses consist of up to 24 5-ml injections of a 5 or 10% brain suspension, equivalent to 6-12 g of brain tissue.

Neurotissue vaccine usage has long been associated with an unacceptably high
incidence of post-vaccinal reactions including inflammation, malaise, and paralysis (Bareggi, 1889). Localised redness, swelling and pruritus commonly occur (Cornwall, 1919); they often appear within several hours of vaccination, reach their maximum within 6 to 8 hours, and are usually gone by the following day. Delayed tuberculin type reactions are less common; they are often associated with malaise, headache, low grade fever, lymphadenopathy, urticaria and nausea, and may herald the development of paralysis (Horack, 1939; Cook et al, 1955). At first, the neurological reactions were considered to be due to the street virus or its modification during the course of treatment (Horack, 1939). This view was challenged by Tonin in 1902 who reported a case of post-vaccinal paralysis with the animal suspected of being rabid remaining healthy (Horack, 1939). Subsequently a number of cases of 'rage de laboratoire' were described in which fixed rabies virus could be recovered from the brains of vaccinees (Remlinger, 1927; Remlinger, 1935; Proca & Bobes, 1940). Marinesco (1908) first suggested that the brain tissue component of the vaccine might itself exert a toxic effect, but only when immune adjuvants became available and brain tissue was adjuvanted did animal experiments establish the encephalitogenic nature of uninfected brain (Rivers et al 1933; Kabat et al, 1947; Kabat et al, 1948; Lumsden, 1949).

Remlinger, at the first international conference on rabies in Paris in 1927, reported 329 neurological reactions among 1,164,264 persons treated (1 per 3,539 treatments), but considered this to be an underestimate and suggested a figure of 1 episode per 500 to 1,000 treatments (Remlinger, 1927). Greenwood (1945) reported 222 cases among 1,290,758 treatments (1 per 5,814 treatments). Review of 22 smaller series indicates the overall incidence of neurological reactions to be 1 in 1180 treatments, a quarter of which terminate fatally (Simon, 1913; Philips et al, 1921; Schweinberg, 1924; Herrmann, 1926; McCoy, 1930; Moftah & Nabih, 1931; Stuart & Krikorian, 1933; Horack, 1939; Yu et al, 1941; Redewill & Underwood, 1947; Sellers, 1947; Pait & Pearson, 1949; Appelbaum et al, 1953; McFadzean & Choa, 1953; Cook et al, 1955; Greenberg & Childress, 1960; Shiraki...
et al, 1962; Chowdhuri et al, 1969; Kuwert, 1970; Kitamoto et al, 1971; Rohmer et al, 1971; Bögel et al, 1975). The risk of developing neural complications is notably higher for persons with a history of allergy (Horack, 1939) or previous rabies treatment (Sellers, 1947). Other factors affecting the incidence include the type of vaccine used (Greenwood, 1945), its neural tissue content (Shiraki et al, 1962), the total number of injections given (Appelbaum, 1953), and presence of phenol-killed bacterial contamination, which behaves as an adjuvant (Svet-Moldavskij et al, 1965). Also children and teenagers are at lower risk than older persons (Remlinger, 1927; Stuart & Krikorian, 1933; McFadzean & Choa, 1953).

Five forms of neurological complications are described, namely - peripheral neuritis, transverse myelitis, acute ascending paralysis, encephalitis, and 'rage de laboratoire' (Remlinger, 1927; Horack, 1939; Wilson, 1967; Hattwick, 1974). Of these dorso-lumbar myelitis is the most common (Remlinger, 1927). They usually develop 8 to 21 days after the first injection (Remlinger, 1927; Horack, 1939; Redewill & Underwood, 1947; Sellers, 1947; Pait & Pearson, 1949; Appelbaum et al, 1953; Briggs & Brown, 1960; Shiraki et al, 1962), but may appear several weeks after the last (Remlinger, 1927; Assis, 1975). About 15 per cent of vaccinees develop EEG abnormalities during treatment (Gibbs et al, 1961), and more than 50 per cent produce antibodies against brain (Kirk & Ecker, 1949; Koprowski & LeBell, 1950).

The animal model for post vaccinal encephalomyelitis provided a method of characterising the encephalitogenic factor and of studying its pathogenesis. Skin testing of animals with post-vaccinal encephalomyelitis suggested that a cell-mediated response might be important (Waksman & Morrison, 1951; Waksman 1956). This view was supported by passively transferring lymphoid cells from sensitized animals, which reproduced the condition (Paterson, 1960; Astrom & Waksman, 1962). By contrast there was no correlation between the disease process and antibody to brain (Astrom & Waksman, 1962). The sensitizing factor
appeared to be associated with myelin since it was absent from the unmyelinated neural tissue of newborn mammals, frog, or fish brain (Kabat et al, 1948). This led to the treatment of vaccine preparations with aromatic hydrocarbons (Bell et al, 1949), low speed centrifugation (Paterson et al, 1953; Hottle & Peers, 1954), ECTEOLA-cellulose chromatography (Thomas et al, 1965), and fluorocarbon (Kaplan & Turner, 1968) in attempts to remove the factor. All were claimed to be successful, but none have been used routinely in vaccine manufacture.

Contrary to expectation, the literature contains only meagre evidence that neurotissue vaccines actually protect laboratory animals. In 1939, Webster published a critical review of all published work performed under relatively controlled conditions. He concluded, on the basis of the small number of animals employed per test, that Pasteur's data on postexposure immunisation of dogs was unsuitable for analysis (Webster, 1939). Only one set of data showed significant postexposure protection, but this was only achieved providing at least 25 per cent of the animals' body weight in vaccine was given in divided doses (Fermi, 1908). The results of later experimental work were similarly considered unimpressive (Rhodes, 1946).

The influence of prophylactic treatment in preventing rabies has been estimated by comparing the mortality in treated and untreated groups of persons bitten by animals causing the death from hydrophobia of one or more subjects or animals. Semple quotes the outcome following bites by two infective dogs (Semple, 1919). Of 18 persons bitten, 9 received treatment and remained well, whereas 7 of the 9 who were untreated died from rabies. According to Veeraraghavan & Subrahmanyan (1958), the mortality from rabies among the completely treated is 6.5 per cent, rising to 20 per cent among the incompletely treated, and 42.1% among the untreated. Thus Semple vaccine apparently saves approximately 84 per cent of those who would otherwise develop rabies and die. Kitamoto et al (1971) followed up 460 people who had been bitten by proven rabid animals and treated with
ultraviolet ray inactivated vaccine. Altogether 4.4 per cent died, but the mortality rose to 10.8 per cent following bites to the head. Another method of assessment is to take instances in which a presumably rabid animal has bitten several persons of whom some accept treatment and others refuse. Cornwall (1923) estimated the mortality among the treated and untreated to be 2.9 per cent and 6.2 per cent respectively. Yu (1941) similarly reports mortalities of 4.5 per cent and 10.0 per cent, suggesting that only one out of every two persons is protected by brain tissue vaccine. A number of reports stress, however, that the mortality after severe bites by 'infective' or rabid wild animals is virtually as high as in individuals who receive no treatment (Nicolic, 1952; Gremliza, 1953; Baltazard & Ghodssi, 1954; Bahmanyar, 1966; Fathi et al, 1970). Thus neuro-tissue vaccine affords limited protection following minor exposures, but is less effective following head bites, and is evidently of little or no value following particularly severe exposures.

3. Neurotissue vaccines prepared from immature brain

The rapid multiplication of fixed rabies virus to high titres in the brains of immature animals, and the relative absence of myelin from their neural tissue, have been exploited in the preparation of potent vaccines from suckling mouse (Fuenzalida & Palacios, 1955), suckling rat (Svet-Moldavskij et al, 1965) and suckling rabbit brains (Gispen et al, 1965). All three vaccines had successful preliminary clinical trials (Fuenzalida et al, 1964; Svet-Moldavskij et al, 1965; Gispen & Saathof, 1965). No cases of post-vaccinal encephalitis were recorded among 16 943 persons treated with suckling mouse brain (SMB) vaccine in Chile (Fuenzalida et al, 1966), or among 9 500 subjects given suckling rat brain vaccine in Russia (Svet-Moldavskij et al, 1965), and no-one died from rabies. SMB vaccine inactivated by ultraviolet light, betapropiolactone, or phenol is the most extensively used treatment in Latin America.

Although originally considered to be free from the encephalitogenic factor, there have been a number of reports of paralytic reactions, mostly Guillain-Barré
syndrome, associated with the use of SMB (Trejos et al, 1971; Held & Adaros, 1972; Assis, 1975; Toro et al, 1977; Escobar et al, 1979), even after a reduced schedule of immunisation (Vergara et al, 1979). Held and Adaros (1972) collected details of 32 cases from 8 countries and estimated the complication rate to be 1 case per 7,865 persons treated. A further 21 cases were reported by Toro et al (1977) who estimated the complication rate to be 1 in 4,615 treatments with a 52 per cent mortality. Thus from the available data, suckling mouse brain vaccine appears to be only marginally safer than vaccines derived from adult neural tissue. Moreover, the extremely large numbers of animals required for its manufacture increases both the problem of harvesting the brains aseptically and the risk of contamination by endogenous viruses (Turner, 1969). Attempts were made to purify SMB vaccine by centrifugation and chromatography (Sikes & Larghi, 1967; Lavender, 1970), but events in the field of tissue culture vaccine have overshadowed these developments.

4. Avian tissue vaccines

Avian tissue vaccines evolved from attempts to avoid neural tissue substrates completely. Two, the Flury low egg passage (LEP) and Flury high egg passage (HEP) vaccines, were prepared in chick embryos and used as live virus vaccines. The Flury strain of rabies virus was originally isolated from a girl of that name (Leach & Johnson, 1940; Koprowski, 1954). It was 'fixed' by serial passage in day-old chicks and then adapted to the developing chick embryo (Koprowski & Cox, 1948). At the low egg passage level (40-50 passages) the virus was innocuous for most mammals when injected parenterally (Koprowski & Black, 1950; Koprowski & Black, 1954; Koprowski et al, 1954). At high egg passage level (~180 passages), it became apathogenic for adult mice, rabbits and dogs when given intracerebrally, but remained lethal for suckling mice and rhesus monkeys (Koprowski, 1954). Flury HEP vaccine was given extensive trials in man (Schwab et al, 1954; Fox et al, 1955; Fox et al, 1957; Sharpless et al, 1957; Ruegsegger et al, 1961,
Schnurrenberger et al, 1961; Ruegsegger & Sharpless, 1962; Tierkel & Sikes, 1967) but with disappointing results. Many grams of infected embryo material were necessary to elicit a humoral response and anaphylaxis and suppuration at the injection site were unacceptable complications (Schwab et al, 1954; Fox et al, 1955; Sharpless et al, 1957). The use of this material was based upon the belief that it underwent limited replication in non-neural tissue in the recipient. Schwab et al (1954) and Sharpless et al (1957) found no evidence to support this hypothesis, so the vaccine had no advantage over inactivated material containing the same quantity of antigen. Moreover the attenuated state was lost upon further passage (Koprowski, 1954), making this live vaccine potentially far more dangerous. Accordingly, the WHO Expert Committee on Rabies, in its 5th Report, recommended that the use of Flury vaccines be restricted to animals (WHO, 1966).

The successful adaptation to growth of fixed strains of rabies virus in embryonated duck eggs (Powell & Culbertson, 1950) permitted the development of an attenuated vaccine for human use (Peck et al, 1955) from tissue possessing little or no encephalitogenic activity (MacFarlane & Culbertson, 1954). The vaccine was subsequently inactivated with beta-propiolactone which had been found to yield vaccine of greater antigenicity than formalin or phenol (Lo Grippo & Hartman, 1955; Peck et al, 1956). According to Greenberg & Childress, (1960) duck embryo vaccine (DEV) evoked humoral responses in man more rapidly than Semple vaccine; seroconversion occurred in about 90% of subjects with either vaccine, but the titres were subsequently greater with Semple vaccine. In contrast to Semple vaccine, DEV produced no EEG abnormalities in man (Gibbs et al, 1961).

DEV became commercially available during the late 1950's and early 1960's and until recently it was used to treat approximately 30,000 people annually in the United States, although its efficacy has never been established by clinical trial. Some data suggests that it conferred no greater immunity than neuro-tissue vaccines (NTV) - between 1957 and 1967, when both DEV and NTV were available in the
USA, there were 6 deaths among 117 700 treated with NTV, and 7 deaths among 172,000 treated with DEV (Sikes, 1969a). There have been conflicting reports on the ability of DEV to evoke antibody responses, some investigators noting good seroconversion (Cabasso et al, 1971; Kuwert et al, 1972), others finding the vaccine to be poorly antigenic, particularly when given with passive immunisation (Crick & Brown, 1970; Ellenbogen & Slugg, 1973; Hattwick et al, 1974; Hattwick et al, 1976). Multiple inoculations were necessary to ensure adequate responses, but in animals and persons receiving repeated daily injections, transition to IgG synthesis is delayed and the IgM response prolonged (Rubin et al, 1971; Grandien & Espmark, 1974; Turner, 1978). In rabies, where neural rather than viraemic spread is important, IgM is of questionable value since it is largely restricted to the intact circulation (Spiegelburg, 1974; Mims, 1976). By contrast IgG antibodies enter the tissues and, unlike IgM, they afford protection to laboratory animals infected with rabies (Turner, 1978; Mifune et al, 1980).

Because of DEV's apparent freedom from serious adverse effects it has been widely recommended for pre-exposure use. The results have generally been disappointing. Even after 3 to 4 doses seroconversion is rarely found in more than 80% of vaccinees and the titres are generally low (Anderson et al, 1960; Dieterich et al, 1961; Schnurrenberger et al, 1961; Farrar et al, 1964; Larsh, 1965; Smith, 1966; Tierkel & Sikes. 1967; Garner, 1976; Morgan et al, 1978). Comparison of the potency of DEV and NTV consistently showed the avian vaccine to be inferior despite having an equivalent protein content (Dean & Sherman, 1962). The concentration of DEV was subsequently increased by 40 per cent (Kissling & Reese, 1963) but this had no appreciable effect (Sikes & Larghi, 1967) except on adverse local and systemic reactions which occurred in 67 per cent of subjects during pre-exposure prophylaxis and in up to 100 per cent during post-exposure treatment (Rubin et al, 1973). The spectrum of reactions included anaphylaxis, occurring in 0.5 to 0.9 per cent of vaccinees, and neurological accidents occurring with an estimated incidence of 1 in 32 600 and fatality rate of 1 in 210 000 (Prussin &
Katabi, 1964; Kaiser et al, 1965; Cowdrey, 1966; Perrine et al, 1968; Harrington & Olin, 1971; Mozar et al, 1973; Rubin et al, 1973). Schlenska (1976) reported two cases of neuroparalytic reactions among an estimated 6 000 to 8 000 vaccinees. According to Schell et al (1980), there have been only 6 neurological reactions among the persons who received the more than 6.5 million doses of DEV produced in Switzerland. Nonetheless, concern regarding both the antigenicity and safety of DEV prompted the same authors to purify and concentrate their product by density gradient centrifugation.

5. **Tissue culture vaccines**

Until recently licensing authorities would only consider primary cultures of embryonic tissue or strains of diploid cells of human origin as being suitable for the preparation of tissue culture vaccines for use in man.

*Primary cell culture vaccines:* The adaptation to growth of rabies virus to hamster kidney cells (Kissling, 1958) permitted the development of experimental rabies vaccines prepared using primary hamster kidney cells as the substrate (Fenje, 1960; Ott & Heyke, 1962a, b; Kissling & Reese, 1963); most batches were of adequate potency and several that were studied contained 20- to 25-fold less protein than NTV or DEV. Nonetheless, low virus yields, slow growth, and the risk of anaphylaxis to serum present in the culture medium made this approach to vaccination seem impracticable. Fenje & Pinteric (1966) overcame these difficulties using a chemically defined medium, concentration of the virus by ultracentrifugation, and aluminium adjuvants. Their vaccine was given clinical trials in 1966 and was licensed in Canada in 1968 for pre-exposure prophylaxis (Fenje, 1974). There are few published reports concerning its use. According to Cho et al (1972) and Fenje (1974), approximately 15 000 doses have been distributed annually with most subjects responding to a three dose schedule of
immunisation. However, doubts were expressed about this vaccine when low or absent titres were found in almost 70 per cent of veterinarians vaccinated during the previous 6 years (Devadason, 1976). In Russia, an ultraviolet light-inactivated vaccine has been prepared from primary hamster kidney cells infected with the Vnukovo-32 strain of rabies virus (Selimov & Aksenova, 1966). By 1978 almost 60 000 persons had each received up to 125 ml of the vaccine for post-exposure prophylaxis. No neuroparalytic reactions were observed and only two cases of rabies developed among those treated (Selimov et al, 1978; Sinnecker, 1978). In Michigan, Garner et al (1976) similarly developed a vaccine in primary hamster kidney cells and reported antibody responses in everyone given a three dose course of pre-exposure immunisation.

Primary chick embryo cells have been used to prepare live adjuvanted HEP Flury virus vaccine (Ruegsegger & Sharpless, 1962) and HEP (Kondo et al, 1974) and LEP (Barth et al, 1983) vaccines inactivated with β-propiolactone. All three products are immunogenic and the inactivated vaccines have been given post-exposure trials in man (Kondo, 1978; Bijok, 1985). Vaccines have similarly been prepared on primary cultures of duck embryo cells (Lavender & Van Frank, 1971; Lavender, 1973), foetal bovine kidney cells (Atanasiu et al, 1974; Atanasiu et al, 1978), canine kidney cells (van Wezel & van Steenis, 1978), and quail embryo cells (Bektimirova et al, 1978). Foetal bovine kidney cell rabies vaccine is licensed for use in France and by 1984 3 000 doses had been administered for pre- and post-exposure use (L'Institut Pasteur Production, 1985).

Vaccines derived from human cell substrates: From the early 1970's to the mid 1980's most attention focused on the vaccine developed at the Wistar Institute in WI-38 human diploid cells (Wiktor et al, 1964; Wiktor & Koprowski, 1965). The cells, which were isolated from human embryonic lung (Hayflick & Moorhead, 1961), have been extensively tested and used for vaccines against polio (Plotkin,
rubella (Plotkin, 1971), cytomegalovirus (Plotkin et al, 1976), and varicella (Takahashi et al, 1975). The virus used was the Pitman Moore 1503-3M strain of fixed rabies virus that was derived from a strain originally isolated by Pasteur and maintained by the National Institute of Health, Bethesda, Md, USA (Wiktor et al, 1978a). In 1962-63 the virus was adapted to growth in WI-38 cells and was propagated in WI-38 cells for 52 passages. Subsequently a master seed pool was prepared in 1965, and the seed virus was transferred to a vaccine-producing facility, l'Institut Merieux, in 1966. The seed strain was distributed to two further vaccine manufacturers, Behringwerke and Wyeth in 1969 and 1971 respectively. The Wyeth product was a sub-unit vaccine that had been inactivated with tri-(n)-butylphosphate. In contrast the vaccines produced by l'Institut Merieux and Behringwerke are both whole virion preparations grown in MRC-5 human diploid lung fibroblasts and inactivated with β-propiolactone. The early batches of the Merieux vaccine, however, were prepared on WI-38 cells. The current European products differ only insofar that the Behringwerke vaccine is concentrated and purified by rate zonal untracentrifugation (Hilfenhaus et al, 1976), whereas the Merieux product is concentrated by ultrafiltration. The American (Wyeth) vaccine was never produced commercially due to manufacturing and potency problems.

Early experimental batches of β-propiolactone-inactivated human diploid cell strain vaccine (HDCSV) were immunogenic in mice (Wiktor et al, 1964) and monkeys (Wiktor & Koprowski, 1965; Sikes et al, 1971a), but because the virus yields from WI-38 cells are relatively poor (Wiktor & Clark, 1975) it was necessary to concentrate the cell supernatants to obtain vaccine of high potency. Using either ultrafiltration through nitrocellulose membrane; continuous zonal centrifugation; or zinc acetate precipitation, desalting on a Sephadex column, and concentration by high-speed centrifugation, it was possible to concentrate the virions present in the tissue culture fluids 100-200-fold and to increase purity to a considerable degree (Sokol et al, 1968; Wiktor et al 1969; Schlumberger et al,
1970; Koprowski, 1971). The Wistar group, in collaboration with CDC, Atlanta, then gave a single dose of concentrated HDCS vaccine to monkeys that had previously been inoculated with street rabies virus (Wiktor, 1971; Sikes et al, 1971a). These investigators found that the new vaccine gave greater protection than 14 daily doses of DEV or a massive dose of homologous antirabies serum and soon afterwards the first human trials were performed on members of the Wistar Institute (Plotkin, 1980).

The first human trial of HDCSV was reported in Lyon in 1972 (Wiktor et al, 1973b; Kaplan 1974). Eight subjects who had received a rabies vaccination previously developed an anamnestic response after a single dose of the Wyeth split-product HDCS vaccine (HDCSV-SP); the new vaccine was given intradermally to seven and intramuscularly to one subject. Intramuscular vaccination of eight other individuals who had not previously received rabies vaccine evoked an antibody response in 5 after the first dose and substantial antibody titres in seven after the second.

During the next few years, the clinical trials with HDCSV were directed towards establishing optimal regimens for pre-and post-exposure immunization and freedom from troublesome reactions. When volunteers were immunised with a 1 ml dose of whole virion HDCS vaccine (HDCSV-WV) produced by l'Institut Merieux, Bahmanyar (1974) found that antibodies were not detectable on day 7, but on days 21 and 35 the titres were similar to those of persons given seven or twelve daily doses of phenolized brain tissue vaccine. The highest levels were seen in groups given four doses of vaccine on days 0,1,2,3, or 0,3,7, and 21, the latter schedule, however, produced the highest levels and a more prolonged response. Nonetheless, administration of 50 IU/kg of mule antirabies serum on day 0 markedly suppressed the antibody response of subjects receiving HDCSV-WV on days 0, 3, 7, and 21. Cabasso et al (1974) found that 1 ml of HDCSV-SP produced only a modest response in previously non-vaccinated subjects, never reaching a level higher than 0.8 IU/ml
over a 56-day testing period. Antibody was not detectable on day 7, either after a single dose, or after two doses given three days apart. The best response - a day 56 geometric mean antibody titre of 6.4 IU/ml - followed 4 doses of vaccine given on days 0, 7, 14, and 28. Overall there was a definite suggestion in the various injection schedules that higher and more sustained antibody levels were reached when the interval between the first and second doses was longest. However, the addition of Al(OH)₃ to the vaccine did not improve its antigenicity.

Despite the absence of the disease in the United Kingdom, rabies occasionally develops in animals held in quarantine, and animal handlers in such facilities should be considered for vaccination. The early reports of HDCSV and the reluctance of Medical Research Council (MRC) animal handlers to receive DEV prompted the initial MRC studies with the new vaccine. The principal issues addressed by the MRC group (Drs David Tyrrell, Lisa Hill, George Turner, and Fred Aoki) was the value of intradermal versus intramuscular vaccination and vaccine acceptability. In 1975 the group reported that 35 subjects, who had not received rabies vaccine previously, all responded to a single intramuscular 1ml dose of HDCSV-WV and to one-tenth of this dose administered intradermally (Aoki et al, 1975). A second dose, given 28 days after the first and by the same route, produced substantial titre elevations in all subjects. The inoculations were well tolerated, although there were more local reactions after intradermal vaccination. A third injection, scheduled for day 56, seemed unnecessary but had already been given to many vaccinees when the rabies antibody tires became available. Accordingly it was decided to expand the study to include groups given one or two 'primary' doses only. I joined the group in October 1975 and replaced Dr Aoki, a visiting research worker from Canada, as clinical coordinator.

Earlier that year, two patients with rabies received intensive medical supportive care in London. A large number of nursing, medical, and auxiliary staff were potentially at risk of exposure, yet only a small amount of vaccine was
available. Rapid immunisation with minimal quantities of vaccine was therefore indicated and an excellent opportunity arose for the MRC group to study the immune response to different rapid dose schedules of HDCSV-WV administered intradermally (ID) in 0.1 ml volumes. A regimen of 4 x 0.1 ml of vaccine given on day 0 into each limb stimulated a more rapid antibody response than 0.1 ml of vaccine given ID on each of days 0, 3, 7, and 14 (Turner et al, 1976). All volunteers had titres greater than 1.7 IU/ml by day 14, and 7 of 10 receiving 0.1 ml into each limb on day 0 had detectable antibody by day 7.

Other reports on HDCSV also appeared in the literature at this stage. Cox & Schneider (1976) confirmed the intradermal route to be highly effective, but they encountered unpleasant reactions with HDCSV-WV upon boosting which led them to abandon this route of immunisation. Garner et al (1976) and Shah et al (1976) reaffirmed the antigenicity and freedom from adverse reactions of HDCSV-SP and HDCSV-WV respectively. By far the most exciting observation, however, was published by Bahmanyar et al (1976) who reported that 45 persons who had all been severely bitten by proven rabid animals in Iran were all protected by one dose (40 IU/Kg body weight) of mule rabies immune serum given as soon after exposure as possible (day 0) and an intramuscular regimen of HDCSV-WV given on days 0, 3, 7, 14, 30, and 90. Much remained to be learnt of the persistence of antibody, booster responses, interferon and cell-mediated responses to HDCSV and of abbreviated regimens with this costly product.

6. Passive Immunization

Studies in animals: As early as 1889, Babes & Lepp demonstrated experimentally the efficacy of antirabies serum. Since then many workers have examined the value of passive immunization. The results, which ranged from complete protection (Fermi, 1909) to none at all (Marie, 1908), are difficult to interpret since they were often based on a small number of experimental animals, control animals were often few or not included, and the titration of rabies antibody with any degree of
reproducibility could not be assured at the time. (Cabasso, 1976). Following improvements in study design, a few workers showed no benefit of serum (Proca et al 1934a; Shortt et al 1935; Covell et al, 1936; Jonessco, 1939), but others obtained consistently good results in various animals, ranging from mice to monkeys, if large doses were given shortly before, during, or after infection (Proca et al 1934b; Hoyt et al, 1935; Hoyt et al, 1936; Hoyt & Gurley, 1937; Yen, 1942; Habel, 1945; Koprowski et al, 1950).

Experiments on combined active and passive immunization were also undertaken. Hoyt et al (1938) reported that a single injection of antiserum, given at the beginning of active immunization, largely nullified the effects of 12 daily doses of vaccine. However, other workers (Habel, 1945; Koprowski et al, 1950; Koprowski & Black, 1954; Habel, 1954) presented experimental evidence in mice, hamsters, guinea pigs, dogs and monkeys that postexposure treatment with vaccine and subcutaneous or intramuscular antiserum would prevent street rabies virus infection following peripheral virus inoculation. Moreover, the protection attained was generally better than that observed after serum or vaccine treatment alone. The difference between these results and those of Hoyt et al (1938) no doubt reflects that the dosage of antiserum is critical particularly when given with a poor immunogen - too small a dose may fail to give adequate antibody levels, and too large a dose may suppress active immunity. Habel's observation (1941) that street rabies virus could be demonstrated up to 72 hours afterwards in the muscle at the site of peripheral inoculation seems at first to have been overlooked, since more than 15 years were to elapse before experimental evidence stressed the importance of prompt infiltration of wounds with antiserum (Gallardo et al, 1957; Schindler, 1961; Soloviev & Kobrinski, 1962; Dean et al, 1963b; Kaplan & Paccaud, 1963). However, a number of earlier studies had suggested the usefulness of local infiltration (Habel, 1954) and in 1957 the World Health Organisation Expert Committee on Rabies recommended its use (WHO, 1957).
The high incidence of serum sickness that accompanies use of heterologous anti-tetanus serum (Moynihan, 1955) and anti-rabies serum (Hosty & Hunter, 1953; Kraliner & Belaval, 1965; Ellenbogen & Slugg, 1973) in man prompted further studies in animals. Some experimental data provide evidence that homologous antibody preparations may provide superior protection when compared with heterologous antibody (Veeraraghavan et al, 1958; Anderson & Sgouris, 1966; Winkler et al, 1969). In contrast, other workers found homologous antiserum to give greater immunosuppression than a heterologous product (Archer & Dierks, 1971). Although the adjunctive effect of antiserum is generally not in doubt, there is abundant evidence that passively administered antibody often prolongs the incubation period but gives little, or no, protection by itself (Marie, 1908; Jonnesco, 1939; Yen, 1942; Koprowski & Black, 1954; Sikes et al, 1971a; Wiktor, 1971; Baer & Cleary, 1972).

Passive immunization - studies in man: Babes & Cerchez first reported the use of antirabies serum in the treatment of man in 1891; 12 persons who were severely bitten by a rabid wolf received in addition to Pasteur's treatment, 4 to 6 injections of hyperimmune whole blood from dogs or man. All 12 survived but an untreated person who was bitten by the same wolf died, indicating that they had all been exposed to an infective animal (Semple, 1908). Marie (1908) reported "most encouraging" results from mixtures of antiserum and virus for the treatment of more than 300 persons. Semple (1908) treated 202 exposures to rabies with equine antiserum and vaccine and reported 3 deaths. These early studies and a succession of others (Shortt et al, 1935; Covell et al, 1936; Proca et al, 1937; Smith, 1937; Koprowski et al, 1950; Koprowski & Cox, 1951) claiming a beneficial effect of sero-vaccine therapy over vaccine treatment alone were flawed by the failure to include vaccine only controls, and/or by neglecting to establish the diagnosis of rabies in the biting animal by laboratory methods.
One of the most rigorous tests of anti-rabies therapy is the post-exposure treatment of persons bitten by proven rabid wolves. The bites are often multiple and deep, and in Iran, despite a course of treatment with NTV, they carry a 25% risk of mortality overall and a 42% mortality among individuals wounded on the head or face (Baltazard & Ghodssi, 1954). It was in this setting that a convincing demonstration of the value of antirabies serum in man was first made. In August 1954, a rabid wolf entered a village in Iran and bit 29 persons, some very severely. Five individuals with head wounds and treated with a course of NTV alone showed no demonstrable antibodies in their sera until the nineteenth day following the start of treatment; 3 of these 5 persons died from rabies. In contrast, 11 of 12 survivors among 13 individuals similarly exposed, who received antirabies serum plus a course of vaccine, had demonstrable antibodies early and throughout an 8 week period of observation (Baltazard et al, 1955; Habel & Koprowski, 1955). Selimov et al (1959), and Fathi et al (1970) have provided further evidence for the beneficial effect of antiserum following bites by rabid animals, mostly wolves. Aggregated data from Baltazard et al (1955), Selimov et al (1959), and Fathi et al (1970) indicate that antiserum saves the lives of approximately 85% of persons who would otherwise develop rabies and die, despite treatment with NTV.

From a series of studies coordinated by WHO it was concluded that antibody was demonstrable no sooner than 7 to 10 days following the start of treatment with NTV, HEP Flury vaccine, or DEV (Atanasiu, et al, 1956; 1957; 1961; 1967). By contrast passively administered antirabies serum given at the start of treatment produced circulating antibody within 24 hours and persisted until the active antibody response became measurable. However, it was also noted that immune serum suppressed the antibody response to vaccination (Atanasiu, et al, 1956; 1957; 1961; 1967; Fox et al, 1957) and that this interference could be overcome by booster injections 10 and 20 days after a 14-day course of inoculations (Atanasiu et al, 1967). Despite the extra protection afforded by passive immunization treatment failures were still reported following combined sero-vaccine therapy.
(Baltazard et al, 1955; Anderson et al, 1966; Bahmanyar, 1966; Chowdhuri et al, 1969; Rubin et al, 1969; Dehner, 1970; Fathi et al 1970; Hattwick et al, 1972b; Shah & Jaswal, 1976). It remains unclear whether these failures were caused by delay in giving the serum, inadequacy of the antibody preparation, low potency of the vaccine, refractoriness of the subject to the antigenic stimulus, or the interfering effect of the serum on vaccine.

Immune globulin preparations of human origin were developed during the late 1950's to overcome the problems of anaphylaxis and serum-sickness that were commonly associated with the use of crude equine preparations (Hosty & Hunter, 1953; Karliner & Belaval 1965; Ellenbogen & Slugg, 1973). Hosty et al (1959) used the methods of Cohn (1946), and Oncley (1949) to fractionate a serum pool prepared from volunteers hyperimmunized with DEV and HEP Flury vaccine. Although the final product was low-titred in comparison with equine antiserum, satisfactory blood levels were obtained after doses of 0.5 ml/Kg and the decrease in titre was more gradual than with the heterologous product (Hosty et al, 1959). This was consistent with the findings of others that homologous tetanus antibody in humans is far superior to equine antibody on a unit basis (Smolens et al, 1961). The subsequent production of human rabies immune globulin (HRIG) of similar or greater potency (Anderson & Sgouris, 1966; Winkler et al, 1969; Sikes, 1969b; Cabasso et al, 1971) was followed by trials to establish the dosage to be used with DEV (Cabasso et al, 1971; Loofboow et al, 1971; Rubin et al, 1973b; Dierks et al, 1974; Hattwick et al, 1974); administration of 15 to 40 IU/Kg of HRIG generally resulted in the early appearance of antibody but inhibited the development of active antibody. Based on these data, the regimen recommended for prophylaxis of severe exposure consisted of 20 IU/Kg combined with 21 doses of DEV given over 21 days and 2 booster injections given 10 and 20 days after completion of the primary series (Hattwick et al, 1976). Despite the large number of doses given, some 10% of persons still had inadequate antibody responses by day 30 to 90 (Hattwick et al,
1976). Much remained to be learnt about the interaction between HDCSV and passively administered homologous and heterologous antibodies.

7 Assessing immunity to rabies

For more than 50 years, the mouse neutralisation test (MNT) devised by Webster & Dawson (1935), has been invaluable in assessing humoral response to rabies vaccines and in establishing optimum regimens for pre- and post-exposure prophylaxis. The test has provided a useful diagnostic tool and remains an important method of assessing vaccines although it suffers with a number of disadvantages, primarily the requirement for large numbers of young adult mice and the facilities necessary to contain them. The test also suffers with an inherent variability making it difficult to correlate results between laboratories, even when standard antiserum is included in each test (Fitzgerald et al, 1975; Kuwert et al, 1978). Moreover, the MNT has the disadvantage of requiring at least 14 days for completion.

To overcome these problems a number of attempts have been made in recent years to develop an in vitro assay for virus neutralising antibody. A cytopathic effect with rabies virus, although reported by some authors (Atanasiu & Lépine, 1959; Fernandes, 1963; Kissling & Reese, 1963; Abelseth, 1964; Wiktor et al, 1964; Yoshino et al, 1966; Sedwick & Wiktor, 1967; Schneider, 1973), has not been sufficiently reliable to permit routine tissue culture neutralisation tests such as inhibition of cytopathic effect or plaque-inhibition. However, the introduction of immunofluorescent staining techniques (Goldwasser & Kissling, 1958; King et al, 1965; Debbie et al, 1972) led to the rapid fluorescent focus inhibition test (RFFIT) which requires only 24 hours for completion (Smith et al, 1973) and correlates well with the MNT (Smith et al, 1973; Cho & Fenje, 1975; Guillemin et al, 1981). Unfortunately the equipment and reagents necessary for the RFFIT are expensive and not widely available in the Third World.

In 1960, Kaplan et al, showed that infection of primary chick embryo cells
with CVS rabies virus subsequently inhibited plaque formation by western equine encephalitis virus. This interference phenomenon has since been confirmed using primary and continuous cell cultures, different strains of rabies virus and a number of challenge virus strains (Cohen et al, 1963; Wiktor et al, 1964; Fernandes et al, 1964; Selimov et al, 1965; Yoshino et al, 1966; Maksumov, 1970; Szegoleit & Gerth, 1971; Kawai et al, 1975; Smith et al, 1977), and provides the basis for an in vitro neutralisation test which I developed following the smallpox incident in Birmingham, and the introduction of more stringent laboratory precautions.

The complement fixation test (Kuwert, 1973a), the haemagglutination inhibition test (Kuwert, 1973b), and mixed haemadsorption test (Grandien & Espmark, 1977) have gained only limited acceptance for determination of rabies antibody, and radioimmunoassay (Wiktorex, 1973b) is expensive, carries some risks, and because it requires expensive equipment, is restricted to central facilities. Enzyme-linked immunosorbent assay (ELISA) has been used in the serodiagnosis of many infectious diseases (Sever & Madden, 1977; Yolken, 1982) and has been proposed as a possible alternative to neutralisation tests for rabies (Atanasiu et al, 1977, Thraenhart & Kuwert, 1977). The ELISA test depends upon two factors: (a) that antigen or antibody can be attached to a solid-phase support yet retain its immunological activity, and (b) that either antigen or antibody can be linked to an enzyme and that the complex retains enzyme-activity. Enzyme-labelled reagents are safe, have a long shelf-life, and yield objective results with the same sensitivity as radio-immunoassay, yet can be used with relatively inexpensive equipment. Accordingly I developed an indirect microplate ELISA method for the detection and measurement of rabies antibody using a modification of the method described by Kraaijeveld et al, (1980) for detection of coronavirus antibodies.

Historically virus neutralizing antibody has been considered to be the key to successful rabies prophylaxis, both before and after virus exposure (Habel, 1945; Koprowski et al, 1950; Habel & Koprowski, 1955; Winkler et al, 1969; Crick, 1973), and inadequate antibody responses to neurovaccine and duck embryo vaccine
have generally been held responsible for occasional treatment failures.

Nilsson et al (1979) challenged immunised mice, selected genetically for 'high' and 'low' antibody responses, and found that 'high' responders were the more resistant to rabies. The possible involvement of other immune systems was not excluded and intracerebral challenge with a fixed strain of rabies virus is a poor model of natural infection. However, review of the literature in which pre-exposure immunisation of various animal species was followed by peripheral challenge with street rabies virus (Koprowski & Black, 1954; Abelseth, 1964; Dean et al, 1964; Cabasso et al, 1965; Fenje & Pinteric, 1966; Sikes et al, 1971a & 1971b; Larghi et al, 1976; Soulebot et al, 1981), reveals a 96% reduction in mortality when antibody was present at the time of challenge. In the absence of antibody, survival was 57.5% if a humoral response had been demonstrated previously, and 23.6% if it had not. Hence the presence of actively induced antibody prior to exposure is evidently associated with protection. Inspection of the antibody titres of the 14 animals that died revealed a range of 1 in 2 to 1 in 1 750 (GMT 1 in 18), implying that even substantial titres of antibody cannot always guarantee protection. Several studies in pre-immunized dogs and cats show a relation between the titre of neutralizing antibody and protection against rabies virus challenge: a neutralizing antibody titre of approximately 0.5 IU/ml at the time of challenge is necessary for uniform protection (Sikes et al, 1971b; Bunn et al, 1984), and this titre is accepted as indicating a satisfactory response to vaccination.

The association between antibody and protection is much less clear for treatment given post-exposure. In several studies in monkeys in which challenge with street virus was followed by a single injection of potent rabies vaccine of tissue culture origin, animals succumbing to infection showed high titered antibody responses comparable in height and time of appearance to those of survivors (Sikes et al, 1971 a, b; Wiktor, 1971; Baer et al, 1979). Habel & Koprowski (1955) and Chowdhuri et al (1969) also showed that the antibody profiles of patients dying after
NTV treatment were equivalent to those of survivors. In addition, many workers have found that passively administered antibody alone may prolong survival but have little influence on mortality (Sikes et al, 1971a; Yen, 1942; Koprowski & Black, 1954; Baer & Cleary, 1972). Possibly in post-exposure treatment interferon induction and cellular immunity have key protective functions.
B. SUBJECTS, MATERIALS AND METHODS
CHAPTER 7.
SUBJECTS, MATERIALS AND METHODS.

SUBJECTS AND MATERIALS

1. Subjects

2. Vaccines

3. Human rabies immune globulin (HRIG)

4. Equine antirabies serum (EARS)

5. Tissue culture materials
   5.1 Sodium bicarbonate
   5.2 Antibiotics
   5.3 L-Glutamine
   5.4 Trypsin EDTA
   5.5 Foetal calf serum
   5.6 Tryptose phosphate broth

6. Buffers
   6.1 0.1M Tris-HCl buffer pH 7.4
   6.2 0.1M HCl-KCl buffer pH 2

7. Tissue culture media
   7.1 Medium 199 with 5% Foetal Calf Serum
   7.2 Medium 199 with 2% Foetal Calf Serum
   7.3 Eagle's Minimal Essential Medium with 10% Foetal Calf Serum
   7.4 Eagle's Minimal Essential Medium with 1% Foetal Calf Serum
   7.5 Glasgow modification of MEM medium : BHK-21 Growth medium
   7.6 Glasgow modification of MEM medium : BHK-21 Maintenance medium
   7.7 25mM Tris buffered maintenance medium
7.8 Carboxymethyl Cellulose - Tyrosine (CMCT) Overlay

8 Cell cultures and their maintenance

8.1 GL-V3 (African Green Monkey kidney) cells
8.2 BHK-21 and BHK-21:13S cells
8.3 L929 cells

9 Virus diluents

9.1 Diluent A
9.2 Diluent B
9.3 Diluent C

10. Cell fixative

11. Cell stains

11.1 Crystal violet
11.2 Fluorescein labelled antirabies conjugates

12. Virus strains

12.1 Rabies virus strains

12.1.1 CVS-11
12.1.2 CVS-11(HK 113)
12.1.3 CVS-11(HK 113, BHK 7)
12.1.4 ERA
12.1.5 ERA-ATTC
12.1.6 LEP Flury virus
12.1.7 Pitman Moore strain of rabies virus
12.1.8 Arctic fox street rabies virus

12.2 Vesicular Stomatitis Virus

13. Reference preparations

13.1 International Standard Antirabies antiserum
13.2 MRC Research Standard B interferon, Human 69/19
13.3 Mouse standard interferon 70/331
14 Animals

14.1 Mice

14.2 Rabbits

15 Solutions, plasticware and reagents for ELISA

15.1 Rabies antigen

15.2 Coating buffer

15.3 PBS - Tween 20

15.4 Substrate and substrate buffer

15.5 Conjugates

15.6 ELISA plates
METHODS

1. Mouse neutralization test
2. Assay of interferon
3. Identification of interferon
4. Interference inhibition test
   4.1 Working concentration of the CVS-11 strain of rabies virus
   4.2 Assay procedure
   4.3 Calculation of antibody titres
5. Immunofluorescent staining
   5.1 Tissue culture preparations
   5.2 Brain tissue smears
6. Rabies enzyme-linked immunosorbent assay (ELISA)
   6.1 Assay procedure
   6.2 Calculation of antibody titre
   6.3 Programming steps
7. Lymphocyte transformation
1. Subjects

The 194 subjects in the pre-exposure prophylaxis study (Study 1), were potentially at risk of exposure to rabies and were recruited from among animal handlers in quarantine facilities, veterinary practice, and research laboratories by Dr Aoki and myself.

To prepare human rabies immune globulin (HRIG) for Study 2, I obtained hyper-immune plasma from nine doctors and nurses at University College Hospital, London, who had received post-exposure prophylaxis with HDCSV, and 7 members of staff at the Ministry of Agriculture Fisheries and Food, Tollworth, who were potentially at risk of rabies exposure and wished to receive HDCSV for pre-exposure prophylaxis.

I enlisted 70 veterinary students at the Royal Veterinary College, London for the sero-vaccine study (Study 2), the interferon study (Study 3), and a lymphocyte transformation study (Study 7), and a further 20 volunteers from the animal handlers at the MRC Clinical Research Centre, Northwick Park, for Studies 4, 5, and 8. The late Professor Kuwert, Institute of Medical Virology and Immunology, University of Essen, Germany, provided sera from 53 trainee laboratory technicians in Essen for Study 6, and Dr J Furlong, British Airways Immunisation Centre, London, provided 70 coded sera from vaccinated and non-vaccinated members of staff and travellers for Study 9.

As a visiting WHO Consultant I enrolled 90 volunteers in Islamabad, Peshawar and Hyderabad for the cold-chain study (Study 11) from members of the Army Medical Corps and the staff and students at the National Institute of Health, Islamabad, Pakistan.

Sera from 88 people were provided by Dr D A Warrell in the first collaborative study (Study 12) with the Wellcome-Mahidol University Unit, Faculty of Tropical Medicine, Bangkok, Thailand, and the Queen Saovabha Memorial Institute of the Thai Red Cross. 49 of the 88 people were patients attending the Queen
Saovabha Memorial Institute, Bangkok, and had very doubtful contact with animals suspected of being rabid, or occasionally with human rabies patients, sufficient to warrant vaccination with Semple vaccine according to the criteria of the Institute. The remaining 39 recruits were healthy volunteers from the staff of the Bangkok Hospital for Tropical Diseases.

I recruited and treated most of the patients in studies 13 and 14 during the second and third collaborative studies with the Wellcome-Mahidol University Unit. Ninety-eight patients with doubtful contact with a rabid animal, but who would otherwise have received Semple vaccine at the Queen Saovabha Memorial Institute in Bangkok, agreed to participate in a study of multi-site intradermal and multi-site subcutaneous immunisation (Study 13). A further 155 patients attending the Queen Saovabha Memorial Institute, Bangkok, were recruited to a rabies post-exposure study (Study 14) after it had been confirmed by the fluorescent antibody test (Dean, & Abelseth, 1973) that the brain of the dog or cat which had bitten them contained rabies antigen.

2. Vaccines

Lyophilised human diploid cell strain vaccine (HDCS-WV) was generously provided by l'institut Merieux, Lyon, France, and Behringwerke, Marburg, Germany. Merieux vaccine was prepared from the supernatants of WI-38 or MRC-5 cells infected with the Wistar rabies strain Pitman-Moore/WI-38-1503-3M; the harvests were concentrated 10-fold by ultrafiltration and then inactivated with β-propiolactone. Behringwerke vaccine was prepared using the same vaccine strain grown on MRC-5 cells. It was purified and concentrated by continuous flow ultracentrifugation.

The antigenic values of the vaccines represent the results of NIH potency tests (Seligman, 1973) carried out by the manufacturers or the results of testing in a multi-centre assay (Wiktor et al, 1978b). All vaccine was stored at 4°C and reconstituted immediately before use, except for vaccine used in the cold-chain
study in Pakistan (Study 11).

5% Semple vaccine was obtained from the Thai Red Cross Society's Queen Saovabha Memorial Institute, Bangkok.

The vaccine potencies were as follows:

<table>
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<th>Study No.</th>
<th>Vaccine &amp; Lot No.</th>
<th>U/ml by NIH test; LD_{50} by Habel test.</th>
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<tr>
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<td>HDCS Lot S 0322</td>
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<td>14</td>
<td>Semple Lot 8</td>
<td>264900 LD_{50}</td>
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* Geometric mean of 13 assays

3. Human rabies immune globulin (HRIG)

HRIG Lot 4912 (Hyperab), with a quoted potency of 150 IU/ml and a gamma globulin content of 16.5 mg per cent, was obtained from Cutter Laboratories (Berkeley, California, USA).

HRIG Lot GR1 was obtained from the Blood Products Laboratory, Lister Institute, Elstree, Herts. It was prepared from high titred plasma obtained from 9 volunteers who underwent plasmapheresis 21 and 28 days after an 0.1 ml booster with HDCS vaccine, and 7 others who were given 0.1 ml of HDCS vaccine on days 0
and 28 and were plasmapheresed on days 49 and 56. Each donation was screened for rabies antibody at a level of 6 IU/ml by Dr GS Turner; all were found to exceed this level. A 9.7 L pool of the donations had a titre of 38 IU/ml. Fraction II was prepared by Dr C Rackam using the cold ethanol fractionation method of Kistler & Nitschmann (1962); 376 mls of immune globulin were obtained after lyophilisation and clarification of the reconstituted product. Two antibody titrations were made and the HRIG was diluted to 10.5 mg per cent; it was titrated twice more and the geometric mean antibody titre (GMT) for all four antibody assays was 541 IU/ml. To allow for wastage each vial was labelled as containing 500 IU in its 1.2 ml contents.

HRIG Lot GR4, with a potency of 1148 IU/ml, was obtained from the Blood Products Laboratory, Lister Institute, Elstree, Herts. All HRIG preparations were stored at 4°C.

4. Equine antirabies serum (EARS)

EARS Lot 55, with a maximal quoted potency of 80 IU/ml, was obtained from Institut Pasteur Production, Paris France.

5. Tissue culture materials

5.1 Sodium bicarbonate

Four point four per cent (w/v) and 7.5% of sodium bicarbonate were prepared in distilled water. Phenol red was added to a final concentration of 0.01%. The solution was gassed with a mixture of 9.0% CO₂ in air for 15 minutes at a flow of 5 litres/minute, autoclaved at 10 lb/sq. in. and stored at 4°C.

5.2 Antibiotics

Streptomycin and benzyl penicillin (sodium salt) were obtained from Glaxo Laboratories Ltd., Greenford, Middlesex. Stock solutions containing 10 000 units/ml and 10 mg/ml were prepared in sterile distilled water.
5.3  **L-Glutamine**

Two hundred millimolar L-glutamine was supplied by Flow Laboratories Ltd.,
Irvine, Scotland, and stored at -20°C until used.

5.4  **Trypsin EDTA (Versene trypsin)**

Trypsin EDTA, containing 0.5g trypsin (1/250) and 0.2g EDTA per litre of modified
Puck's saline A were obtained from Gibco, Paisley, Scotland.

5.5  **Foetal calf serum**

This was obtained from Flow Laboratories Irvine, Scotland and stored at -20°C in
20 ml aliquots.

5.6  **Tryptose phosphate broth**

This was obtained from Difco Laboratories Ltd., Detroit, Michigan, USA. It was
prepared to a concentration of 0.295% using 29.5 g in 1000 ml distilled water. It
was autoclaved at 15 lb for 15 minutes at 121°C.

6.  **Buffers**

6.1  **0.1M Tris-HCl buffer pH 7.4**

This was made up using:

- 1.0 M HCl 100 ml
- 1.0 M Tris (121.1g/L) 117.8 ml
- Deionized water 782.2 ml
6.2 0.1M HCl-KCl buffer pH 2

This was made up using:

- 0.2 M HCl 65 ml
- 0.2 M KCl (14.9 g/L) 435 ml
- Deionized water 500 ml

7. Tissue culture media

7.1 Medium 199 with 5% Foetal Calf Serum

This medium was used for growth of GL-V3 cells, and prepared using 199 Medium obtained from Flow Laboratories Ltd., Irvine, Scotland. The medium was prepared as shown below:

- Medium 199 184 ml
- Foetal calf serum 10 ml
- NaHCO₃ (4.4%) 4 ml
- Penicillin (10,000 U/ml) + Streptomycin (10mg/ml) 2 ml

The medium was stored at 4°C until use.

7.2 Medium 199 with 2% Foetal Calf Serum

This medium was used for maintenance of GL-V3 cells and was prepared as shown below:

- Medium 199 190 ml
- Foetal calf serum 4 ml
- NaHCO₃ (4.4%) 4 ml
- Penicillin (10,000 U/ml) + Streptomycin (10mg/ml) 2 ml

The medium was stored at 4°C until required.
7.3 Eagle’s Minimal Essential Medium with 10% Foetal Calf Serum

This medium was used for growth of L 929 mouse cells and was prepared using Eagle’s MEM 10x supplied by Gibco Laboratories, Paisley, Scotland, and contained the following:

- Distilled water 770 ml
- Eagle’s Minimal Essential Medium (10x) 100 ml
- Foetal calf serum 100 ml
- NaHCO₃ (4.4%) 20 ml
- Penicillin (10,000 U/ml) + Streptomycin (10mg/ml) 10 ml

The medium was stored at 4°C until use.

7.4 Eagle’s Minimal Essential Medium with 1% Foetal Calf Serum

This medium was used for maintenance of L 929 mouse cells and contained the following:

- Distilled water 860 ml
- Eagle’s Minimal Essential Medium (10x) 100 ml
- Foetal calf serum 10 ml
- NaHCO₃ (4.4%) 20 ml
- Penicillin (10,000 U) + Streptomycin (10mg) 10 ml

The medium was stored at 4°C until use.
7.5  **Glasgow modification of MEM medium : BHK-21 Growth medium**

This was used for growing BHK-21 cells and was prepared using 10x concentration of Glasgow modified Eagle's medium

- Distilled water: 628 ml
- Eagle's Minimal Essential Medium (10x): 100 ml
- Foetal calf serum: 100 ml
- Tryptose phosphate broth: 100 ml
- Sodium Bicarbonate Solution (4.4%): 62 ml
- Penicillin (10,000 U) + Streptomycin (10mg): 10 ml

The medium was stored at 4°C until use.

7.6  **Glasgow modification of MEM medium : BHK-21 Maintenance medium**

This was used for maintenance of BHK-21 cells and was prepared using 10x concentration of Glasgow modified Eagle's medium:

- Distilled water: 748 ml
- Eagle's Minimal Essential Medium (10x): 80 ml
- Bovine serum albumin (3%): 100 ml
- Sodium Bicarbonate Solution (4.4%): 62 ml
- Penicillin (10,000 U) + Streptomycin (10mg): 10 ml

The medium was stored at 4°C until use.

7.7  **25mM Tris buffered maintenance medium**

This was prepared using 10 mls of stock 1M Tris-HCl buffer and 390mls of maintenance medium. It was used in rabies interference experiments at the Lister Institute where CO₂ facilities were not available.
7.8 Carboxymethyl Cellulose - Tyrosine (CMCT) Overlay

This was prepared using sodium salt, low viscosity, carboxymethyl cellulose obtained from BDH Chemicals, Poole, England and L-Tyrosine. 618 mg of L-Tyrosine was dissolved overnight at 37°C in 1L ion-exchange purified water. 0.75 g carboxymethyl cellulose was added to 25 ml L-Tyrosine solution and left to dissolve overnight at 4°C before autoclaving at 15 lb for 15 minutes. The stock CMCT solution was kept at 4°C until use. The overlay was prepared as follows:-

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eagle's Minimal Essential Medium (1x)</td>
<td>43 ml</td>
</tr>
<tr>
<td>Stock CMCT</td>
<td>25 ml</td>
</tr>
<tr>
<td>Eagle's Basal Medium (2x BME)</td>
<td>25 ml</td>
</tr>
<tr>
<td>Penicillin (10,000 U) + Streptomycin (10 mg)</td>
<td>1 ml</td>
</tr>
<tr>
<td>L-Glutamine (200 mM)</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

The medium was stored at 4°C until use. It was used for GL-V3 and Vero cells; L 929 cell overlay was similar, but contained only MEM (10 mls of 10 x concentrate), no BME.

8 Cell cultures and their maintenance

8.1 GL-V3 (African Green Monkey kidney) cells

This line was obtained from the cell bank at the Clinical Research Centre, Northwick Park, and was originally derived from the kidneys of a vervet monkey (Christofinis, 1970). The cells were propagated into 20 oz tissue culture glass medical flats in a growth medium consisting of 199 medium with 5% foetal calf serum; they were split 1:4 when required. This cell line was used for interferon assays, the interference inhibition test, and studies of rabies virus interference.

8.2 BHK-21 and BHK-21:13S cells

BHK-21 (Macpherson & Stoker, 1962) and BHK-21:13S cells, originally derived
from the kidneys of baby hamsters, were obtained from the cell bank at the Center for Disease Control, Atlanta, Ga and were used for growing rabies virus. The split ratio used was 1:6.

8.3  **L929 cells**

L929 cells, originally derived from normal subcutaneous areolar and adipose mouse tissue (Sandford et al 1948), were used in tests of the specificity of interferon-like activity, and were obtained from the cell-bank at the Clinical Research Centre, Northwick Park Hospital. The cells were propagated in 20 oz tissue culture glass medical bottles; the split ratio was 1:3.

All three cell lines were propagated at 37°C in a humidified atmosphere containing 5% CO₂. For maintenance of cell lines in the laboratory, confluent monolayers were subcultured. This was carried out as follows:-

The growth medium was decanted and the cell monolayers washed twice with PBS; 2-3 mls of trypsin or versene-trypsin solution were than added, and distributed over all the cell sheet by rocking the bottle for 30 seconds. The trypsin was then decanted, and the cells incubated at 37°C for 3 - 5 minutes until they had detached from the glass surface. Cells were then dispersed by vigorous pipetting and suspended in growth medium to the required concentration. Monolayer cultures were maintained with maintenance medium which was changed every three days.

9  **Virus diluents**

Diluents A, B, and C were used for tissue culture and animal inoculation experiments:

9.1  **Diluent A**

This was used for diluting vesicular stomatitis virus (VSV) and serum specimens for
interferon:-

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eagle's Minimal Essential Medium (1x)</td>
<td>191 ml</td>
</tr>
<tr>
<td>Penicillin (10,000 U) + Streptomycin (10mg)</td>
<td>2 ml</td>
</tr>
<tr>
<td>L-Glutamine (200 mM)</td>
<td>2 ml</td>
</tr>
<tr>
<td>NaHCO₃ (4.4%)</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

9.2 **Diluent B**

This was used as the serum diluent for the mouse neutralisation test and as the rabies virus diluent for intracerebral inoculation. It was prepared using 0.75% bovine serum albumin (Bovine albumin powder, Fraction V, Armour Pharmaceutical Company Ltd.) in PBS A containing 100μg/ml di-ethylaminoethyldextran of approximate molecular weight $2 \times 10^6$ (Lot Numbers. 0-5876 and 107B-1190) from the Sigma Chemical Company.

9.3 **Diluent C**

This was prepared using three volumes of diluent A and one volume of diluent B.

10. **Cell fixative**

Formol saline [(10% v/v) formalin (40% w/v formaldehyde) in 0.85% NaCl] was prepared using the following:-

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>17 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1800 ml</td>
</tr>
<tr>
<td>Formalin</td>
<td>200 ml</td>
</tr>
</tbody>
</table>

The formalin was added to the saline solution and the resulting solution stored at room temperature.
11. **Cell stains**

11.1 *Crystal violet*

This was prepared by dissolving crystal violet stain (Colour index 42555) in industrial methylated spirits and then adding water in the following amounts:-

- **Crystal violet** 1 g
- **Industrial methylated spirits** 200 ml
- **Water** 800 ml

The resulting solution was filtered through filter paper and stored at room temperature.

11.2 *Fluorescein labelled antirabies conjugates.*

Rabies antinucleocapsid conjugate obtained from the Institut Pasteur, Paris was used in studies in England to quantitate rabies virus infection of cell monolayers. It had been prepared by immunization of rabbits with purified nucleocapsids from the Pasteur strain of fixed rabies virus. Rabies conjugate provided by CDC Atlanta was used at the CDC to quantitate infection of cell monolayers and to detect rabies antigen in organs from experimental animals. It was supplied as a lyophilised horse-immune globulin conjugated with fluorescein isothiocyanate.

12. **Virus strains**

12.1 *Rabies virus strains*

12.1.1 **CVS-11**

CVS-11, with a titre of $10^7 \text{LD}_{50}$ per ml, was supplied by Dr TJ Wiktor, Wistar Institute, Philadelphia, to Dr GS Turner, Lister Institute, Elstree, in whose laboratory the virus was used. After two further passages in BHK-21 cells at the Center for Disease Control, Atlanta, Ga, USA, the virus pool V 1220 A had a titre of $10^{8.5} \text{LD}_{50}$ per ml.
12.1.2 CVS-11(HK 113)

CVS-11(HK 113) was kindly provided by Miss SP Bauer, Center for Disease Control, Atlanta, Ga, USA. It was originally derived from the strain adapted to primary hamster kidney cells by Kissling (Kissling, 1958). After two passages in BHK-21 cells virus pool V 1224 A had a titre of $10^{7.38} \text{LD}_{50}$ per ml.

12.1.3 CVS-11(HK 113, BHK 7)

CVS-11(HK 113, BHK 7) was obtained from Miss SP Bauer, Center for Disease Control, Atlanta, Ga, USA. After two further passages in BHK-21 cells virus pool V 1223 A had a titre of $10^{7.13} \text{LD}_{50}$ per ml.

12.1.4 ERA

ERA virus was originally supplied by Dr TJ Wiktor to Dr GM Baer at the CDC Lawrenceville Facility, Lawrenceville, Ga, USA. After two passages in BHK-21 cells virus pool V 1222 A had a titre of $10^{7.75} \text{LD}_{50}$ per ml.

12.1.5 ERA-ATTC

ERA-ATTC provided by Dr GM Baer, was originally obtained from the American Type Tissue Collection, Rockville, Maryland, USA and had a titre of $10^{8.13} \text{LD}_{50}$ per ml.

12.1.6 LEP Flury virus

LEP Flury virus was provided by Prof E Kuwert, Institute for Medical Virology and Immunology, Essen, Germany. After two passages in BHK-21 cells virus pool V 1221 A had a titre of $10^{6.38} \text{LD}_{50}$ per ml.

12.1.7 Pitman Moore strain of rabies virus

The Pitman Moore strain of rabies virus was provided by Miss J Crick,
Animal Virus Research Institute, Pirbright, Surrey. Virus pool V1219 A was prepared after two passages in BHK-21 cells and had a titre of $10^{8.13}$ LD$_{50}$ per ml.

12.1.8 Arctic fox street rabies virus

Arctic fox street rabies virus was a gift from Dr GM Baer. One- to two-day old mice were inoculated with a $10^{-3}$ dilution of a salivary gland suspension obtained from the CDC collection at Lawrenceville. After death at 11-12 days, the mice were stored at -70°C and a 20% suspension of brain aspirate was made in a 4% bovine serum albumin. This material was centrifuged, aliquoted and stored at -70°C. An initial 1 in 2 dilution was made to give a $10^{-1}$ suspension of brain which had a titre of $10^{5.13}$ MlLD$_{50}$ per ml in suckling mice and 50 LD$_{50}$ per ml in rabbits when inoculated intranuchally in two locations.

12.2 Vesicular Stomatitis Virus

The Indiana strain of vesicular stomatitis virus (VSV) was a gift from Miss J Crick, Animal Virus Research Institute, Pirbright, Surrey, and Dr J Obijeski, Center for Disease Control, Atlanta, Ga, USA.

All virus stocks were stored at -70°C.

13. Reference preparations

13.1 International Standard Antirabies antiserum

The international standard antirabies serum was supplied by the Statens Seruminstitut, Copenhagen. The number of international units (IU) in the preparation has been arbitrarily fixed at 80 per ml of lyophilised serum. It was reconstituted with 8 mls of 50% glycerol solution and 0.15 ml volumes containing 10 IU/ml were dispensed and stored at -20°C.
13.2 MRC Research Standard B interferon, Human 69/19

The MRC Research Standard B interferon, Human 69/19, provided by the National Institute for Biological Standards and Control, Hampstead, London, was prepared from human leucocytes infected with Sendai virus. Following a multicentre study, the lyophilised 1 ml contents of each vial were given a potency of 5 000 units. The lyophilised material was reconstituted with 1 ml buffered Hank's solution containing 0.5% BSA. After being diluted 10-fold in Eagle's Minimal Essential Medium, a stock 1 in 20 dilution was made in Diluent A; aliquots containing 25 IU/ml were stored at -70° C.

13.3 Mouse standard interferon 70/331

The mouse standard interferon 70/331 was kindly provided by the National Institute for Biological Standards and Control, Hampstead, London.

14. Animals

14.1 Mice

Dr GS Turner (Lister Institute, Elstree) titrated rabies neutralising antibody using 4 - 6 week Swiss white mice (TO strain) from the animal colonies at the Clinical Research Centre, Northwick Park Hospital. I used 1 - 2 day-old suckling white mice from laboratory colonies at the US Center for Disease Control (CDC), Atlanta, Ga for virus titration studies at the CDC.

14.2 Rabbits

White New Zealand rabbits weighing approximately 8lb were obtained from laboratory colonies at the Lawrenceville Facility, US Center for Disease Control (CDC), Atlanta, Ga., for a post-exposure protection study.
15. **Solutions and Reagents for ELISA**

15.1 *Rabies antigen*

The rabies antigen was kindly supplied by Dr Barth, Behringwerke AG, D-355 Marburg/Lahn, West Germany. It was a whole virus preparation of the Pitman Moore strain of rabies virus, grown in MRC-5 cells and concentrated and purified by continuous flow ultracentrifugation (Hilfenhaus et al, 1976). It was inactivated with β-propiolactone and had an antigenic potency of 269 IU/ml when measured by the antibody binding test (Barth et al, 1981). Electron microscopy, kindly carried out by Mrs Alyne Harrison, CDC Atlanta, revealed approximately $10^{10}$ bullet-shaped whole virions per ml with no disrupted material or host cell material. Certain rabies ELISAs were carried out using a purified rabies antigen that I prepared at CDC Atlanta.

15.2 *Coating buffer*

An 0.05M sodium carbonate-bicarbonate buffer (pH 9.6) containing 0.02% sodium azide, was obtained as a 10-fold concentrate by Don Whitley Scientific, Shipley, Yorkshire. It was stored at 4°C in a tightly sealed container.

15.3 *PBS-Tween 20*

Phosphate buffered saline containing 0.05% Tween 20 (PBST) was obtained as a 10-fold concentrate from Don Whitley Scientific, Shipley, Yorkshire. The solution was used to dilute test and control sera and to wash the plates after each stage of the test; it was stored at room temperature.

15.4 *Substrate and substrate buffer*

Phosphatase substrate, consisting of an 0.1% solution of p-nitrophenylphosphate disodium in 10% (wt/vol) diethanolamine buffer (pH 9.8) with 0.02% sodium azide and 0.01% MgCl$_2$.6H$_2$O, was prepared using Sigma 104 5 mg tablets.
containing p-nitrophenyl-phosphate disodium (Sigma London Chemical Company, Poole, Dorset) and substrate buffer obtained from Don Whitley Scientific Ltd. The substrate was freshly prepared before each assay (using one 5mg substrate tablet to each 5 ml of substrate buffer) and protected from light.

15.5 Conjugates

Goat immunoglobulin G directed against human IgG, Goat immunoglobulin G directed against human IgM, and rabbit immunoglobulin G directed against mouse IgG were conjugated with alkaline phosphatase and were purchased from Miles Laboratories, Ltd., Stoke Poges, Slough.

15.6 ELISA plates

Boxes of 50 96-well, flat-bottomed, immulon microelisa plates, M129A, were obtained from Dynatech Laboratories, Billinghamurst, Sussex.

METHODS

1. Mouse neutralization test

Virtually all rabies neutralizing antibody titrations for the MRC studies were carried out by Dr GS Turner at the Lister Institute, Elstree, using the mouse neutralization test (MNT) (Atanasiu, 1973). Test sera were inactivated for 30 minutes at 56°C and serial 5-fold dilutions were prepared in phosphate buffered saline containing 0.75% bovine serum albumin. The CVS-11 challenge virus was diluted in phosphate buffered saline with 0.75% bovine serum albumin to contain 20-30 LD₅₀ per 0.03 ml of serum-virus mixture. To determine the actual quantity of virus used in the test, fivefold dilutions of challenge virus were mixed with an equal volume of diluent containing 20% horse, heat-inactivated serum. The
International Standard antiserum to rabies virus was titrated with each batch of sera using a two-fold dilution series known on the basis of previous studies to bracket the 50% endpoint dilution \([ED_{50}]\) value. After incubation for 90 minutes, all the tubes were placed in a vessel filled with crushed ice. Batches of 4 - 6 week old mice weighing 14 - 16 g each were then inoculated intracerebrally with 0.03ml of each dilution, using 5 mice per dilution. The different groups of mice were placed separately in labelled boxes and kept under observation; a record was made of those dying between the 6th and 20th days. Endpoint titres of the International Standard antiserum, the serum under test and the \(LD_{50}\) actually used in the test were calculated using the Spearman-Kârber method (Lorenz & Bögel, 1973). To express the potency of test sera in IU/ml, the differences between the logarithms of the \(ED_{50}\) of the test and standard sera were calculated. Because the International Standard antiserum was kept at a concentration of 10 IU per ml, the potency of test sera in IU/ml were calculated from:

\[
10 \times \text{antilog}_{10} (\log_{10} ED_{50} \text{ standard serum} - \log_{10} ED_{50} \text{ standard serum})
\]

2. **Assay of interferon**

Interferon was titrated using a modification of a plaque-inhibition technique (Merigan, 1971), using a semi-micro method (Zisman & Merigan, 1973) in which GL-V3 cells, a monkey kidney cell line responsive to interferon (Matthews & Lawrence, 1979) were grown in 96-well disposable plastic 'Linbro' trays. The 96 wells in the plastic trays were each seeded with 1.0ml growth medium containing \(10^5\) GL-V3 cells. Confluent monolayers were formed after 24 - 48 hours incubation at 35 - 35°C in a humidified atmosphere containing 4 - 5% \(CO_2\). The sera were diluted 1:10, 1:31, 1:100 in diluent A and added to at least 2 monolayers and held overnight at 37°C in a \(CO_2\) incubator. The fluid was removed and the cells washed with maintenance medium and then challenged by adding 30 - 60 plaque forming units (pfu) of vesicular stomatitis virus (VSV) to each well. After a 90
minute adsorption period, the 0.2 ml of virus inocula were replaced with 1.0 ml quantities of carboxymethylcellulose overlay containing tyrosine (CMCT). The monolayers were incubated a further 36 to 48 hours, then fixed and sterilized with formol saline and stained with crystal violet. Parallel tests with dilutions of 5, 2, 1, 0.5, and 0.1 U/ml of the MRC Research Standard B 69/19 human leucocyte interferon were included in all assays. A reduction in plaque count below the 95 or 99% confidence limits of control counts (Lorenz, 1962) was regarded as indicating the presence of interferon, and the titre of this activity was calculated by reference to the titration of the standard.

3 Identification of interferon

Because most sera contained only low titres of interferon-like activity, tests for specificity were generally restricted to effects on non-primate cells, and a plaque-inhibition assay similar to that described above was performed on mouse L929 cells using VSV and mouse standard interferon 70/331. To further identify the antiviral activity as interferon, a pool of high titred samples underwent the following treatments:

a) Centrifugation at 105 000g for 2 hours at 4°C using the 10 x 10 Ti angle-head rotor in an MSE Superspeed 65 at 38 000 rpm.

b) Acid treatment. Samples were dialysed against an 0.1 M KCl-HCl buffer (pH2) for 24 hours at 4°C; the pH was returned to 7.4 by treatment with an 0.1M Tris HCl buffer at room temperature.

c) Heat treatment. Samples were heated in a water bath at 56°C for 2 hours, and

d) Trypsin treatment. Sensitivity to trypsin treatment was carried out using an
0.5% trypsin solution in the sample to be tested. 0.05 ml of 0.25% trypsin was added to 0.2 ml of the sample and incubated at 37°C for 90 minutes. 0.02 ml of 0.2% soybean trypsin inhibitor was then added.

Specimens were then assayed for interferon as described previously.

4 Interference Inhibition test

Pressure placed upon the limited facilities at the Lister Institute by new safety regulations, and the large number of sera requiring titration led to the development of an in vitro assay of rabies neutralizing antibody. In Chapter 8, I describe the experiments leading to the development of the 'Interference Inhibition Test'. The steps involved in the assay, which evolved from the interferon assay described previously, are described below:

4.1 Working concentration of the CVS-11 strain of rabies virus

To determine the working concentration of the CVS-11 strain of rabies virus used in the test, monolayers of GL-V3 cells in 96-well disposable 'Linbro' plastic trays were inoculated with 0.2 ml of 0.5 log₁₀ dilutions of virus in diluent C and were incubated at 34°C in a humidified atmosphere containing CO₂. Maintenance medium (1.0 ml) was added to each well and the plates were incubated for 4 days after which the medium was removed and wells containing both rabies-virus infected and non-infected cells were challenged with 0.2 ml of VSV containing 30-50 pfu. After 1 hour at 34°C the VSV challenge was replaced with 1.0 ml CMCT overlay and the monolayers were incubated for a further 2 days before fixing in formol saline and staining with crystal violet. Rabies virus (0.2 ml) containing 10⁵ LD₅₀ ml⁻¹ reliably caused complete inhibition of VSV plaque formation and was used for subsequent inhibition tests (Fig 7.1).
Fig. 7.1 Inhibition of VSV plaque formation in monolayers of GL-V3 cells by CVS-11 rabies virus. Cell monolayers were infected with 0.5 log_{10} dilutions of CVS-11 and challenged after 96 hours with 30 p.f.u. VSV/well.
4.2 Assay procedure

Undiluted test sera were inactivated at 56°C for 60 minutes, diluted 1:10 in diluent A and then serially in two-fold increments. Equal volumes of rabies virus diluted in diluent B to give a final concentration of $10^5$ LD$_{50}$ ml$^{-1}$ were added to dilutions of test sera and international standard antiserum. The samples were mixed by gentle agitation and incubated at 37°C for 60 minutes. Each serum-virus mixture (0.2ml) was inoculated into GL-V3 monolayers as described above. Control monolayers were inoculated with 0.2 ml rabies virus to ensure that complete inhibition of VSV plaque formation occurred. After 90 minutes adsorption and 4 days' incubation, the test and at least six wells of untreated cells per plate were infected with VSV. The mean plaque count of the latter was used for the determination of the 50% plaque reduction point.

4.3 Calculation of antibody titres

Table 7.1 shows the results of a typical titration of the international standard antiserum which was maintained as a working stock containing 10 I.U. ml$^{-1}$.

Table 7.1: Titration of the international standard antiserum by the interference inhibition test

<table>
<thead>
<tr>
<th>Serum dilution</th>
<th>No. of plaques/well</th>
<th>Mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 : 50</td>
<td>40 51 42 56</td>
<td>48.25</td>
</tr>
<tr>
<td>1 : 100</td>
<td>55 42 48 40</td>
<td>46.25</td>
</tr>
<tr>
<td>1 : 200</td>
<td>51 54 51 38</td>
<td>48.5</td>
</tr>
<tr>
<td>1 : 400</td>
<td>24 34 36 40</td>
<td>33.5</td>
</tr>
<tr>
<td>1 : 800</td>
<td>4  0  2  0</td>
<td>1.5</td>
</tr>
<tr>
<td>1 : 1600</td>
<td>0  0  0  0</td>
<td>0</td>
</tr>
<tr>
<td>1 : 3200</td>
<td>0  0  0  0</td>
<td>0</td>
</tr>
<tr>
<td>1 : 6400</td>
<td>0  0  0  0</td>
<td>0</td>
</tr>
<tr>
<td>VSV plaque counts}</td>
<td>46 49 48 50 }</td>
<td></td>
</tr>
<tr>
<td>in control wells }</td>
<td>62 59 59 59 }</td>
<td>52.6</td>
</tr>
<tr>
<td>}</td>
<td>48 50</td>
<td></td>
</tr>
</tbody>
</table>
Plaque reduction (50%) calculated from the control titration in this example was 26.3 plaques/well and the serum dilution at which 50% reduction occurred was between 1:400 and 1:800. Exact end-points were determined by plotting plaque counts on the arithmetic axis against serum dilution on semi-logarithmic graph paper. The intercept of the line representing 26 plaques with that plotted for the counts at each serum dilution indicates the 50% end-point. In this example, the value was 1:420. The titres of other serum samples were determined similarly. Titres in I.U. were calculated by reference to the international standard antiserum.

5 Immunofluorescent staining

5.1 Tissue culture preparations

Fluorescent antibody staining of tissue culture preparations was carried out using the method described by Wiktor (1973a). Coverslips or slides with infected cells were washed in phosphate buffered saline (PBS) and fixed in acetone held at -20°C for 30 minutes. After fixation they were dried and the monolayers covered with a few drops of hyperimmune fluorescein conjugated anti-rabies horse globulin. The preparations were incubated for 45 minutes at 37°C and then washed for 5 minutes in two changes of PBS and one change of distilled water and dried.

5.2 Brain tissue smears

In Thailand these were initially confirmed as rabies-positive by Dr Chiewbamroongkiat after a preliminary screen for Negri bodies. Duplicate brain smears were examined by Dr Mary Warrell at a later date. Both used the technique of Dean & Abelseth, (1973). Briefly, small sections of brain tissue (Ammon's horn, cerebrum, or cerebellum) were cut and placed on a wooden tongue depressor, cut surface facing upwards. A clean microscope slide was then touched against the cut surface of the section and pressed gently downwards with just enough pressure to create a slight spread of the exposed surface of the tissue against the glass slide.
Several impressions were usually made on one slide. The specimens were then fixed in acetone, dried and stored at -20°C and stained as described above. Positive and negative controls were included in each assay.

6 Rabies enzyme-linked immunosorbent assay (ELISA)

6.1 Assay procedure

Rabies IgG and IgM antibodies were detected using a modification of the method described by Kraaijeveld et al (1980), for detection of coronavirus antibodies. Wells of flat-bottomed microelisa plates were coated with 0.2ml amounts of rabies antigen diluted 1:200 in 0.05M sodium carbonate-bicarbonate buffer (Elisa coating buffer, pH 9.6) containing 0.02% sodium azide. Plates were then covered with parafilm and incubated overnight at room temperature (21°C). After incubation, plates were washed four times with phosphate buffered saline containing 0.05% Tween 20 (PBST) and shaken dry.

The test and control sera were absorbed overnight with equal volumes of foetal calf serum (0.3ml volumes) and serial fivefold dilutions, usually in the range 1/20 to 1/12 500, were prepared in PBST. 0.2ml of each dilution were added to the wells of the antigen-coated plates and together with suitable controls were incubated for 3 hours at room temperature. After incubation, the plates were washed four times in PBST and shaken dry. In most assays, goat immunoglobulin G, directed against heavy and light chains of human IgG and conjugated with alkaline phosphatase, was diluted 1 in 800 and added in 0.2ml volumes to each well of the plate and left overnight at room temperature. After four additional washes with PBST, 0.2ml of phosphatase substrate, consisting of a 0.1% solution of p-nitrophenylphosphate disodium in 10% (Wt/Vol) diethanolamine buffer (pH 9.8) with 0.02% sodium azide and 0.01% MgCl₂.6H₂O, was added to each well. The times at which the first and last wells received substrate was recorded, the time half-way between the two was "time 0". The plate was incubated at room temperature and
absorbence values were read after 30 minutes at 405 nm in a Flow Laboratories Multiskan spectrophotometer.

6.2 Calculation of antibody titre

During most assays, test sera and 8 control sera from unimmunised subjects were serially diluted fivefold. End-point titres of antibody were determined using a programmable TI-58 Texas Industries pocket calculator. This was programmed to determine the intercept (x) between the quadratic curve fitted to the optical density (OD) values of dilutions of test sera, and the straight line fitted to the mean plus 2.5 standard deviations of the mean OD values of appropriate negative controls. From this intercept, the titre of antibody (X) was calculated (Figure 7.2). The calculation involves two formulae:

\[
\text{intercept } x = 2 + \frac{1}{B} \left[ E - \sqrt{E^2 - 2B(Y_2 - D)} \right]
\]

and

\[
\text{endpoint titre of test sample } X = d_1 \cdot n \cdot x^{-1}
\]

where

\[B = Y_1 - 2Y_2 + Y_3\]

\[D = (S_1 + S_2 + S_3)/3\]

\[E = (S_3 - S_1 - Y_3 + Y_1)/2\]

\[Y_1, Y_2, Y_3 \text{ are OD values of the test sample at dilutions } d_1, d_2, d_3\]

\[S_1, S_2, S_3 \text{ are OD values (mean + 2.5 SD) for eight known negative samples at dilutions } d_1, d_2, d_3\]

\[d = \text{the denominator of the first dilution (}d_1\text{) used in the calculation (e.g., 500)}\]

\[n = \text{the dilution factor}\]
Fig. 7.2  Intercept of quadratic curve fitted to optical density (OD) values of dilutions of test sera, and the straight line fitted to the mean plus 2.5 standard deviations of the mean of OD values of appropriate negative controls.
6.3 Programming steps

To programme the TI-58 press [Pgm] followed by the keystrokes listed in the following sequence:

<table>
<thead>
<tr>
<th>Step and key code</th>
<th>Key sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>000</td>
<td>76 [2nd] [Lbl]</td>
</tr>
<tr>
<td>001</td>
<td>11 [A]</td>
</tr>
<tr>
<td>002</td>
<td>42 [Sto]</td>
</tr>
<tr>
<td>003</td>
<td>01 [1]</td>
</tr>
<tr>
<td>004</td>
<td>42 [Sto]</td>
</tr>
<tr>
<td>005</td>
<td>02 [2]</td>
</tr>
<tr>
<td>006</td>
<td>91 [R/S]</td>
</tr>
<tr>
<td>007</td>
<td>42 [Sto]</td>
</tr>
<tr>
<td>008</td>
<td>03 [3]</td>
</tr>
<tr>
<td>009</td>
<td>65 [X]</td>
</tr>
<tr>
<td>010</td>
<td>02 [2]</td>
</tr>
<tr>
<td>011</td>
<td>95 [=]</td>
</tr>
<tr>
<td>012</td>
<td>22 [INV]</td>
</tr>
<tr>
<td>013</td>
<td>44 [SUM]</td>
</tr>
<tr>
<td>014</td>
<td>01 [1]</td>
</tr>
<tr>
<td>015</td>
<td>91 [R/S]</td>
</tr>
<tr>
<td>016</td>
<td>44 [SUM]</td>
</tr>
<tr>
<td>017</td>
<td>01 [1]</td>
</tr>
<tr>
<td>018</td>
<td>22 [INV]</td>
</tr>
<tr>
<td>019</td>
<td>44 [SUM]</td>
</tr>
<tr>
<td>020</td>
<td>02 [2]</td>
</tr>
<tr>
<td>021</td>
<td>91 [R/S]</td>
</tr>
<tr>
<td>022</td>
<td>42 [Sto]</td>
</tr>
<tr>
<td>023</td>
<td>04 [4]</td>
</tr>
<tr>
<td>024</td>
<td>22 [INV]</td>
</tr>
<tr>
<td>025</td>
<td>44 [SUM]</td>
</tr>
<tr>
<td>026</td>
<td>02 [2]</td>
</tr>
<tr>
<td>027</td>
<td>91 [R/S]</td>
</tr>
<tr>
<td>028</td>
<td>44 [SUM]</td>
</tr>
<tr>
<td>029</td>
<td>04 [4]</td>
</tr>
<tr>
<td>030</td>
<td>91 [R/S]</td>
</tr>
<tr>
<td>031</td>
<td>44 [SUM]</td>
</tr>
<tr>
<td>032</td>
<td>04 [4]</td>
</tr>
<tr>
<td>033</td>
<td>44 [SUM]</td>
</tr>
<tr>
<td>034</td>
<td>02 [2]</td>
</tr>
</tbody>
</table>
To complete the programme press [Pgm], then run through test data to ensure that the programme has been entered correctly:

<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
<th>Enter</th>
<th>Press</th>
<th>Display</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Enter Y₁</td>
<td>[1.7]</td>
<td>A</td>
<td>1.7</td>
</tr>
<tr>
<td>2</td>
<td>Enter Y₂</td>
<td>[0.8]</td>
<td>[R/S]</td>
<td>1.6</td>
</tr>
<tr>
<td>3</td>
<td>Enter Y₃</td>
<td>[0.25]</td>
<td>[R/S]</td>
<td>0.25</td>
</tr>
<tr>
<td>4</td>
<td>Enter S₁</td>
<td>[0.3]</td>
<td>[R/S]</td>
<td>0.3</td>
</tr>
<tr>
<td>5</td>
<td>Enter S₂</td>
<td>[0.29]</td>
<td>[R/S]</td>
<td>0.29</td>
</tr>
<tr>
<td>6</td>
<td>Enter S₃</td>
<td>[0.31]</td>
<td>[R/S]</td>
<td>2.863805441 = x</td>
</tr>
<tr>
<td>7</td>
<td>Enter d₁</td>
<td>[2500]</td>
<td>[R/S]</td>
<td>50197.7332 = X</td>
</tr>
</tbody>
</table>

In this example, the titre X is equal to 1 : 50 198. If this figure is displayed the programme has been entered correctly. Continue by entering test Y, S, and d-values using the key sequence [ A ] to initial each new set of data as shown in the example above.

NOTE: This programme is only for a serial dilution factor of 5. For other dilution factors, the key sequence [ 5 ] at location 074 should be changed to the new dilution factor i.e., [ 2 ], [ 4 ], [ 10 ], etc.,

The TI-58 calculator is no longer available. However, endpoint titres can readily be calculated using the following programme written in Microsoft Basic:

```
10 PRINT "TYPE A COMMA AFTER EVERY VARIABLE"
20 PRINT "PRESS 'RETURN' AT END OF EVERY INPUT LINE"
30 PRINT ""
```
119

40 PRINT "ENTER INITIAL DILUTION AND DILUTION FACTOR"
50 PRINT "NOTE! THESE WILL BE CONSTANT FOR THE RUN OF THE PROGRAM"
60 INPUT D, N
70 DATA D, N
80 RESTORE 70
90 PRINT "ENTER VALUES S1,S2,S3"  
100 INPUT S1,S2,S3
110 PRINT "DO YOU REQUIRE THESE 'S' VALUES TO BE USED" 
120 PRINT "AGAIN IN THIS RUN OF THE PROGRAM?"
130 PRINT "TYPE '1' FOR YES, '2' FOR NO"
140 INPUT T
150 IF T = 2 GOTO 180
160 DATA S1,S2,S3
170 RESTORE 160
180 PRINT "ENTER VALUES Y1,Y2,Y3"
190 INPUT Y1,Y2,Y3
200 LET B = Y1 -2*Y2 +Y3
210 LET C = (S1 +S2 + S3)/3
220 LET E = (S3 - S1 -Y3 +Y1)/2
230 LET F = E^2 -2*B*(Y2-C)
240 IF F >0 GOTO 270
250 PRINT "TITRE > DILUTION 3"
260 GOTO 310
270 LET G = 2 + (1/B*(E -SQR(F)))
280 LET X = D * N^G-1)
290 PRINT "THE ANSWER IS" 
300 PRINT X
310 PRINT "HAVE YOU MORE DATA?"
320 PRINT "TYPE '1' IF YOU NEED ENTER ONLY 'Y' VALUES"
330 PRINT "TYPE '2' IF YOU NEED ENTER BOTH 'S' AND 'Y' VALUES"
340 PRINT "TYPE '3' IF YOU WISH TO FINISH"
350 INPUT Q
360 IF Q = 1 GOTO 180
370 IF Q = 2 GOTO 90
1000 END
7 Comparative studies of vaccine potency by ELISA

An ELISA was used to titrate mouse rabies IgG antibody in pooled sera collected 21 days after intraperitoneal inoculation of groups of 12 male, 5-week-old Swiss TO mice with 0.5 ml of 5-fold dilutions of HDCSV in PBS, in the range 1 in 10 to 1 in 6,250. Twenty mice were inoculated with 0.5ml of PBS as control. Each pool was given serial 5-fold dilutions in the range 1 in 20 to 1 in 12 500 using two wells per dilution on two ELISA plates. The negative control sera were inoculated into 6 wells per plate using the same dilutions. Titres of antibody in the serum pools were estimated using a calculator pre-programmed to determine the intercept between the curve fitted to dilutions of the test sera, and the straight line fitted to twice the mean of optical density values of appropriate dilutions of the pooled negative control serum. Regression analysis was then used to calculate the dilution of vaccine giving an antibody titre of 1 in 100 in mice.

8 Lymphocyte transformation

Lymphocyte transformation studies were kindly performed by Dr Peter Cole, Host Defence Unit, Brompton Hospital, London. To prepare lymphocyte populations, four aliquots of 5ml of 'preservative-free' heparinised venous blood from each volunteer were centrifuged at 500 g for 40 minutes with 4ml aliquots of a freshly prepared mixture of Ficoll and Triosil obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. The cell band was harvested, washed, and resuspended to a concentration of $10^6$ lymphocytes/ml in RPMI-1640 medium with HEPES buffer (N-2 hydroxyethylpiperazine-N'-2-ethane sulphonic acid; final concentration, 20mM) modified with 0.5% bicarbonate, 0.292 mg of L-glutamine/ml and 10% human serum. The cells were 95% viable as performed by the trypan blue exclusion test. Enriched T-lymphocyte populations were obtained by taking a thrice washed mononuclear suspension and passing it twice through a nylon-fibre column in a syringe (Greaves and Brown, 1974). The cell suspension obtained contained less than 2% B-lymphocytes as judged by staining with polyvalent fluorescein-labelled
anti-human immunoglobulin reagent. B-lymphocytes were purified by sedimentation of rosettes formed between T-lymphocytes and sheep red blood cells (Greaves & Brown, 1974). Lymphocytes from human cord blood and an unimmunized subject were used as controls. HDCS rabies virus vaccine, Lot Nos. S0203 and R0220, were exhaustively dialysed against PBS and adjusted to the original volume with PBS for use as antigen. Purified grade phytohaemagglutinin (PHA), supplied by Wellcome Laboratories, Beckenham, Kent, was used as the control mitogen.

Five replicate cultures containing 10μl of antigen or mitogen and 200μl of cell suspension containing 10^6 lymphocytes per ml were established in microtitre plates. PHA cultures were harvested after incubation for 72h and antigen cultures after 144 hours at 37°C in a humidified atmosphere of 5% CO₂ in air. Each culture was treated with 10μl of [³H] thymidine (specific activity, 1μCi; Radio-Chemical Centre, Amersham) for 24 hours before harvesting. The cultures were harvested on filter paper discs with use of a Dynatech Minimash machine; the discs were counted at room temperature (about 21°C) in 1 ml of NE 233 scintillation fluid in insert vials and counted in a β counter to obtain counts per minute (cpm) values corrected for background and quenching. The stimulation increment was calculated from the difference between test cultures and control cultures without mitogen or antigen.
C. THE STUDIES
CHAPTER 8.
RABIES VIRUS INTERFERENCE AND THE INTERFERENCE INHIBITION TEST

1. Development of the interference inhibition test (IIT)

To show that the CVS-11 strain of rabies virus induces interference in GL-V3 monkey kidney cells, I inoculated 25μl quantities of virus diluted in PBS containing 0.75% (w/v) BSA and 0.1% di-ethylaminoethyl-dextran (DEAED) into wells of disposable plastic trays containing cell monolayers and 1-ml of growth medium. After 4 days incubation at 34°C in a humidified atmosphere containing 5% CO₂, infected and control monolayers were challenged with dilutions of VSV. After a further 2 days at 34°C, the monolayers were fixed with formol saline, stained with crystal violet, and the number of plaques were counted. The results in Table 8.1 clearly show that monolayers infected with the CVS-11 strain of rabies virus became resistant to superinfection compared to uninfected controls. Furthermore,

<table>
<thead>
<tr>
<th>Inoculum of CVS-11 (LD₅₀/25μl)</th>
<th>Mean plaque count in cell monolayers challenged with log₁₀ dilutions of VSV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10⁻¹</td>
</tr>
<tr>
<td>25 000</td>
<td>0</td>
</tr>
<tr>
<td>2 500</td>
<td>4.5</td>
</tr>
<tr>
<td>250</td>
<td>*</td>
</tr>
<tr>
<td>25</td>
<td>*</td>
</tr>
<tr>
<td>0</td>
<td>*</td>
</tr>
</tbody>
</table>

Note: * - number of plaques too numerous to count
the extent of this interference is apparently dependent upon the titre of the CVS-11 inoculum. These observations prompted an attempt at preventing interference by incubating a fixed quantity of the rabies virus inoculum with dilutions of the international standard antiserum.

Equal volumes of CVS-11 and serial dilutions of antiserum were mixed together and incubated at 37°C for one hour; 25μl of each serum-virus mixture, or rabies virus alone were inoculated into the medium of 6 cell monolayers and the experiment then continued as described above. The results (Table 8.2) show almost complete neutralisation of CVS-11 interference of VSV plaque formation by antiserum. However, the large well-to-well variation in plaque count that occurred.

Table 8.2 Neutralisation of interference: incubation of CVS-11 with dilutions of the international standard antiserum for rabies for 1 hour.

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>VSV plaque count:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
</tr>
<tr>
<td>Monolayers inoculated with VSV only.</td>
<td>55 - 63</td>
</tr>
<tr>
<td>Monolayers inoculated with CVS-11, then challenged with VSV</td>
<td>0</td>
</tr>
<tr>
<td>Monolayers inoculated with serum-virus mixture, then challenged with VSV; titre of international standard IU/ml: 0.1</td>
<td>53 - 64</td>
</tr>
<tr>
<td>0.05</td>
<td>50 - 58</td>
</tr>
<tr>
<td>0.01</td>
<td>46 - 58</td>
</tr>
<tr>
<td>0.005</td>
<td>52 - 64</td>
</tr>
<tr>
<td>0.001</td>
<td>27 - 41</td>
</tr>
<tr>
<td>0.0005</td>
<td>9 - 37</td>
</tr>
<tr>
<td>0.0001</td>
<td>1 - 8</td>
</tr>
</tbody>
</table>

Note: - Wells containing GL-V3 monolayers and 1ml maintenance medium were inoculated with 2 500 LD\textsubscript{50} of virus or serum-virus mixture in an inoculum of 25μl. After 5 days' at 35°C, the medium was removed and the cells challenged with VSV. * - represents a significant reduction (p ≤0.01) in plaque count from the VSV controls.
when the monolayers were inoculated with partially neutralized virus, e.g., at an antibody titre of 0.0005 IU/ml, suggested that an alternative method of virus inoculation should be evaluated. The well-to-well variation was reduced considerably, and the interface between interference and interference inhibition became more pronounced when I inoculated 0.2 ml volumes of serum-virus mixture directly onto the monolayers and allowed the mixture to adsorb for 90 minutes before the addition of 1 ml of maintenance medium (See Table 7.1, Chapter 7). In this and all subsequent experiments, an 0.2 ml inoculum containing $2 \times 10^4$ LD$_{50}$ CVS-11 in 0.0025 to 0.01% DEAED completely inhibited plaque formation by $\leq 100$ pfu VSV when the cell monolayers were challenged 96 hours later. Trypan blue exclusion tests showed the viable cell count per well to be $1.5 \times 10^5$ at the time of rabies virus infection, giving a multiplicity of infection (m.o.i.) of 0.13 LD$_{50}$ per cell for complete interference to occur. This method, which is fully described in Chapter 7, was then adopted for the following studies.

2. **Assessment of observer error**

Plaque counts of the international standard and 93 serum samples were plotted independently by two observers and the calculated titres of each sample were compared to assess observer error. Of the results 96% fell within the zone of a twofold difference from the line of perfect correlation (Figure 8.1). A correlation coefficient of 0.98 with a standard error of 0.1 indicate the low degree of observer error.

3. **Reproducibility of the interference inhibition test**

Of 31 samples retitrated on separate occasions, 26 (84%) repeat titrations fell within the zone of a twofold difference from the line of perfect correlation (Figure 8.2). The high degree of reproducibility was shown by the correlation coefficient of 0.91 and standard error of 0.18. Repeated titrations of the international standard antiserum also demonstrated the reproducibility of interference inhibition. Titres
Fig. 8.1 Correlation between rabies neutralizing antibody titres obtained by independent observers using the interference inhibition test (IIT). Zone of twofold difference (within the area bounded by the broken lines) from the line of perfect correlation (continuous line). Correlation coefficient 0.98, standard error 0.1, number of observations (n) = 93.
Fig. 8.2 Correlation of neutralizing antibody titres in serum samples retitrated by the IIT. Zone of twofold difference (within area bounded by broken lines) from the line of perfect correlation (continuous line). Correlation coefficient 0.91, standard error 0.18, n = 31.
were expressed as $\log_{10}$ of the reciprocal of the serum dilution causing a 50% reduction in plaque counts ($\log_{10} SN_{50}$). In 10 titrations of the international standard antiserum the range of $\log_{10} SN_{50}$ titres was 2.56 - 3.26 with a geometric mean of 2.94 and a standard deviation of 0.25 (Table 8.3).

Table 8.3 Repeat titrations of the international standard antiserum in the interference inhibition test - dilution of the international standard causing a 50% reduction in VSV plaque counts.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Dilution</th>
<th>Log10 of reciprocal dilution ($\log_{10} SN_{50}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LI 1</td>
<td>1/460</td>
<td>2.66</td>
</tr>
<tr>
<td>LI 2</td>
<td>1/360</td>
<td>2.56</td>
</tr>
<tr>
<td>LI 3</td>
<td>1/420</td>
<td>2.62</td>
</tr>
<tr>
<td>LI 6</td>
<td>1/1600</td>
<td>3.20</td>
</tr>
<tr>
<td>LI 7</td>
<td>1/1800</td>
<td>3.26</td>
</tr>
<tr>
<td>LI 8</td>
<td>1/1400</td>
<td>3.15</td>
</tr>
<tr>
<td>LI 10</td>
<td>1/1200</td>
<td>3.08</td>
</tr>
<tr>
<td>LI 11</td>
<td>1/740</td>
<td>2.87</td>
</tr>
<tr>
<td>LI 21</td>
<td>1/950</td>
<td>2.98</td>
</tr>
<tr>
<td>LI 22</td>
<td>1/1100</td>
<td>3.04</td>
</tr>
</tbody>
</table>

4. **Comparison of the interference inhibition test and MNT**

Quantitative correlation was found between the results obtained either by interference inhibition or by MNT (Figure 8.3). Out of 94 sera either positive or negative by both tests there was complete correlation in 93 (99%), only one sample was found to be positive by the IIT but negative by the MNT. The titres computed from the MNT were consistently higher; in 69 (73%) of the samples tested, the MNT titres were greater than or equal to those by the IIT, and in 39 (41%) the titres exceeded a twofold difference. This difference was most apparent with the higher titred samples and may reflect differences in dilution error (serial twofold dilutions
Fig. 8.3 Zone of two- and fourfold differences from the regression line (solid line) correlating rabies neutralizing antibody measured by the IIT and MNT.

Correlation coefficient 0.84, standard error 0.1, number of observations (n) = 94.
for the IIT compared with fivfold dilutions for the MNT). Nevertheless, 63% of the
results fell within the zone of a twofold difference and 96% within the zone of a
fourfold difference from the line of linear regression missing the origin (Snedecor &
Cochran, 1967). The agreement between the two methods was highly significant
(correlation coefficient 0.84, standard error 0.1).

5. Comparative studies of interference
For the comparative studies of interference, monolayers of rabies infected cells in
96-well disposable plastic plates were tested for resistance to approximately 50
p.f.u. VSV as described previously. Strain-dependent differences in the ability to
induce interference were regularly observed. Neither the ERA strain provided by
the Wistar Institute or the ERA-ATCC strain obtained from the American Type
Culture Collection induced interference (Figure 8.4; panel f); both strains
produced some cytopathic effects, and immunofluorescent staining showed that they
had infected the monolayers extensively. By contrast, partial interference was
induced by the CVS-11 (HK-113) and CVS-11 (HK-112, BHK-7) strains [which
were both derived from a strain adapted to primary hamster kidney (HK) at the
Center for Disease Control (Kissling, 1958)] and by the LEP Flury strain provided
by the late Professor Kuwert, Essen, F.R.G., (Figure 8.4; panels c, b, e). This
partial interference was dose-related and also occurred at low multiplicities of
infection with CVS-11 (5th passage level (Figure 8.4; panel a) and the Pitman
Moore (PM) strain, 2nd passage level, (Figure 8.4; panel d). Increasing the m.o.i.
of these strains to 0.1 to 4 LD50 per cell gave a peak of complete interference
followed by partial interference at m.o.i.'s > 4 LD50 per cell.

Autointerference by defective interfering (DI) particles may have been
responsible for the partial interference observed in monolayers inoculated at a high
m.o.i. (≥ 4 LD50 per cell). The CVS-11, PM and LEP Flury strains were therefore
passaged under conditions favourable for the accumulation of DI particles. After
Fig. 8.4 Percentage inhibition of VSV plaque count by monolayers inoculated 96h earlier with increasing $\log_{10}$ MOI(LD$_{50}$/cell) of different strains of rabies virus. 
(a) CVS-11 (•, 5th passage level in BHK-21 cells; o, 8th passage level); (b) CVS-11 (HK-112, BHK-7: 2nd passage level); (c) CVS-11 (HK-113: 2nd passage level); (d) Pitman Moore (•, 2nd passage level in BHK-21 cells; o, 8th passage level); (e) LEP Flury (•, 2nd passage level; o, 5th passage level; (f) ERA and ERA-ATCC strains of rabies virus (3rd passage level).
three serial passages in BHK-21 cells, using undiluted virus inocula for each passage, the harvests were tested for their ability to induce interference. As shown in Figure 8.4; panel a, d, e) interference was not inhibited, but was enhanced by further passage.

No interference was observed when cell monolayers were inoculated with a mixture containing 100 p.f.u. VSV and up to 40 LD50 per cell of the CVS-11 strain. Although not entirely excluding the possibility, the results of this experiment strongly suggest that this type of interference does not occur as a result of competition for common virus receptor sites. Table 8.4 shows that significant interference [when the VSV plaque count was below the 95% confidence limit of the mean count in the controls (Lorenz, 1962)] first became apparent when the monolayers were challenged with VSV 15 hours after infection with the CVS-11 strain at an m.o.i. of 13 LD50 per cell. With the exception of monolayers inoculated

Table 8.4 Percentage inhibition of VSV at different times after infection with CVS-11 rabies virus.

<table>
<thead>
<tr>
<th>CVS-11 m.o.i. (LD50/cell)</th>
<th>Percentage inhibition at time (h):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12</td>
</tr>
<tr>
<td>130</td>
<td>64</td>
</tr>
<tr>
<td>40</td>
<td>66</td>
</tr>
<tr>
<td>13</td>
<td>92</td>
</tr>
<tr>
<td>4</td>
<td>65</td>
</tr>
<tr>
<td>1.3</td>
<td>0</td>
</tr>
<tr>
<td>0.4</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>0.04</td>
<td>0</td>
</tr>
<tr>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td>0.004</td>
<td>0</td>
</tr>
<tr>
<td>0.001</td>
<td>0</td>
</tr>
<tr>
<td>0.0004</td>
<td>0</td>
</tr>
</tbody>
</table>
with the CVS-11 strain at an m.o.i. ≥13 LD₅₀ per cell, which never became completely resistant to VSV, the degree of interference was related to both the incubation period and the m.o.i. of CVS-11. In a similar experiment, challenge of monolayers infected with CVS-11 with an increasing m.o.i. of VSV revealed that the interference could be partially overcome, and when challenged with ≥3×10⁵ p.f.u. VSV/monolayer (about 2 p.f.u./cell) all of the cells showed a cytopathic effect and the monolayers were completely destroyed.

6. Interference, immunofluorescence, and rabies virus yield
Confluent GL-V3 cell monolayers in petri dishes were inoculated with CVS-11 at an m.o.i. of 0.2; after a 3 hour adsorption period the monolayers were washed twice and re-fed with maintenance medium. Cover-slip cultures were similarly prepared and inoculated with CVS-11. At 5, 24, 30, 48, 54, 72, and 96 hours after virus inoculation the supernatants were removed for virus titration, the monolayers were challenged with about 100 p.f.u. of VSV per culture (0.5 x 10⁶ cells), and the cover-slip cultures were fixed and stained with fluorescein-conjugated anti-nucleocapsid antiserum. Thirty hours after infection, specific immunofluorescent staining showed intracellular viral antigen in 62% of 100 high-power fields (x100); this was associated with a 48% inhibition of a VSV plaques and the release of 10²·₂ LD₅₀/ml of CVS-11 into the cell supernatants. By 48 hours, 100% inhibition of VSV had occurred, the virus yield had increased 100-fold, and although the number of high-power fields positive for rabies antigen had not increased, both the size of individual foci and the intracellular content of antigen were observed to be greater. By 96 hours, the yield of infectious virus had increased a further 40-fold to 10⁵.₈ LD₅₀/ml, and 84% of high-power fields had become positive. These observations suggest that the heterologous interference was not associated with any inhibitory effect upon the replication of the inducing virus.
7. **Interference, interferon, and rabies virus yield**

To study this further, a comparison was made of the 96-hour virus yields from monolayers in Corning 25-cm² flasks (Corning, New York) containing about $2 \times 10^6$ GL-V3 cells that were inoculated with $0.5 \log_{10}$ dilutions of the CVS-11 strain, or the non-interfering ERA strain of rabies virus. After a 90 minute adsorption period the monolayers were washed once with 25 IU of antiserum in 10 ml maintenance medium for 10 minutes at 35°C, and then twice with maintenance medium alone. The monolayers were then re-fed with 10 ml of maintenance medium. 96 hours later the flask contents were freeze-thawed, centrifuged to remove cellular debris, and titrated for infectivity in 2-4 day-old mice. Apart from autointerference which occurred in monolayers inoculated with CVS-11 at an m.o.i. of $\geq 16$ LD$_{50}$/cell, there was no significant difference in the yields of infectious virus from monolayers infected with the CVS-11 or ERA strains (Figure 8.5).

Samples from each flask were screened for interferon-like activity; 1 in 2 and 1 in 5 dilutions were made in antiserum (2.5 iu/ml) to prevent live virus from inducing interference. None of the supernatants from cells infected with the ERA strain possessed antiviral activity. By contrast, low titres of interferon (1 to 2 units/ml) were detected in the supernatants from partially resistant and fully resistant monolayers inoculated with the CVS-11 strain at an m.o.i. of $\geq 0.5$ LD$_{50}$/cell; however, no interferon was demonstrable from other fully resistant monolayers inoculated with a lower m.o.i. Thus, like Kaplan et al, (1960), it was not possible to explain the extent of the antiviral activity by the low titres of interferon that were found.

The ubiquitous nature of the interferon system makes it necessary to differentiate its contribution from that of other types of interference. Pretreatment of cell monolayers with actinomycin D inhibits cellular RNA synthesis and may be used to distinguish between intrinsic and interferon-mediated heterologous interference (Marcus, 1977). Accordingly, GL-V3 cell monolayers were pre-
Fig. 8.5 Virus yield from monolayers inoculated with 0.5 log_{10} dilutions of ERA (solid line) and CVS-11 (broken line). Greatest m.o.i. CVS-11 = 160 LD_{50}/cell, ERA 135 LD_{50}/cell.
treated with 0.5μg actinomycin D in 1 ml of maintenance medium for 1 hour; they were then washed and inoculated with log_{10} dilutions of the CVS-11 strain. Fortyeight hours later, the monolayers were challenged with about 40 pfu VSV and the plaque counts compared with those in control monolayers. The results showed actinomycin D to have no inhibitory effect on interference. On the contrary, it enhanced the reduced levels of interference that were observed with monolayers inoculated with CVS-11 at an m.o.i. of ≥4 LD_{50}/cell. Thus, the inhibition of interferon synthesis may have enhanced both the replication of rabies virus and the resistance to superinfection with VSV.

8. Discussion
Cohen et al (1963), while following up the original observations of rabies-induced interference by Kaplan et al (1960), found that the interference inhibition phenomenon was unsuitable for measuring rabies neutralising antibody because the susceptibility of primary chick embryo cells to rabies virus infection and WEE plaque-formation varied considerably. I did not find this to be a problem in the present studies (Nicholson et al, 1979; Nicholson et al, 1981) using an established cell line and a rabies virus strain adapted to tissue culture. The results show that the interference inhibition phenomenon can readily be used for a highly reproducible assay for rabies neutralizing antibody.

Several factors, however, may explain why the titres were higher when the mouse neutralization test was used. First, twofold dilutions were used in the IIT and fivefold dilutions were used in the MNT which may introduce greater error. Second, five mice were used per dilution in the MNT whereas 50 to 60 pfu were used in the IIT with little variability in the plaque counts between wells. Furthermore, the quantity of rabies virus used is greater in the IIT than in the MNT and the avidity of antibody to virus may also be different in the two systems.

The IIT takes only 5 - 6 days to perform, it is relatively inexpensive, the
results are easy to interpret and it could therefore be considered as a suitable alternative to the MNT and the RFFIT. To reduce the number of sera requiring titration by the MNT, the IIT was used for titrating virus neutralizing antibody in a study of lymphocyte transformation following HDCSV. The results are presented in Chapter 10.

The study of rabies virus-infected cells in tissue culture may provide valuable information on the mechanisms underlying both the prolonged incubation periods of the disease and aberrant forms of infection. Persistent infections with rabies virus can be established in a variety of mammalian cells in tissue culture: no cytopathic effect is observed and such cultures can be maintained by cell transfer for many passages. The mechanism(s) underlying the maintenance of a carrier type of infection remains unclear. Kawai et al (1975) isolated a fraction containing defective interfering (DI) particles from the supernatants of cultures persistently infected with rabies virus; no interferon could be detected and the monolayers resisted challenge with homologous virus only. By contrast, Fernandes et al (1964) and Wiktor & Clark (1972) established persistent infections in cultures which resisted challenge with heterologous virus and they were unable to consistently detect interferon.

Rabies infected cells of human (Wiktor et al, 1964), rabbit (Fernandes et al, 1964), viper and lizard (Wiktor & Clark, 1972) origin have all been shown to resist heterologous virus challenge in the absence of detectable interferon. The results of the studies reported here are consistent with intrinsic interference as the mechanism underlying heterologous interference in GL-V3 cells. As regards the differing abilities of CVS-11 and ERA strains to induce interference, this may be analogous to the failure of the RA 27/3 strain of rubella virus to induce intrinsic interference in WI-38 cells (Kleiman & Carver, 1977). Moreover, VSV is among the various unrelated viruses that may be inhibited by this means (Rott et al, 1972; Hunt & Marcus, 1974). Although intrinsic interference blocks VSV replication, it does not inhibit cell killing (Marcus, 1977); this could explain why the
interference induced by the CVS-11 strain could be completely overcome by increasing the m.o.i. of VSV to about 2 pfu per cell.

Whatever the mechanism underlying this type of heterologous interference, there was no evidence that it was associated with any subsequent inhibition of rabies virus replication or dissemination \textit{in vitro}. Also, as the incubation periods are longer and the development of rabies is much less certain in laboratory animals inoculated with the ERA strain, compared to those inoculated with the CVS-11 strain, the development of interference by these strains \textit{in vitro} cannot be related to the evolution of disease \textit{in vivo}. The results of these studies clearly show that the CVS-11 and PM strains are the most efficient inducers of interference in GL-V3 monkey kidney cells and that they are the most suitable strains for the IIT. The ability to induce interference may vary from cell line to cell line, however, and it is recommended that different strains of virus should be tried when developing an IIT in cell lines other than GL-V3.
1. Introduction

As an indirect result of an incident in Birmingham involving smallpox virus, the virus laboratory at the Lister Institute, Elstree, was closed for work with live rabies virus. Although permission was kindly granted for Dr Turner to titrate the outstanding sera from Study 1 (see Chapter 10) by the MNT at the Ministry of Agriculture, Fisheries and Food Laboratories in Weybridge, it was evident that an alternative test utilizing inactivated antigen would be required for future projects. Enzyme-linked immunosorbent assay (ELISA) was being used in the sero-diagnosis of many infectious diseases (Sever & Madden, 1977) and had been proposed as a possible alternative to neutralisation tests for rabies (Atanasiu et al, 1977, Thraenhart & Kuwert, 1977). This chapter considers the development and initial assessment of an indirect microplate ELISA method for the detection and measurement of rabies antibody.

I used dilutions of HDCS vaccine as antigen during preliminary studies, but the results were unsatisfactory, so I tried a highly concentrated, purified antigen preparation from Behringwerke. To determine the optimal concentrations of antigen and conjugate, chequer-board titrations were carried out using human sera positive and negative for rabies antibody. Having established the optimal conditions, the method described in Chapter 7 was then used to evaluate the test and measure rabies antibodies in sera from studies in the UK, Thailand, and Pakistan.
2. Development of the assay

While developing the test I found that the optical density (OD) measurements of sera that were negative for rabies antibody were subject to well-to-well variation and, more importantly, to considerable person-to-person variation. For example, the mean of OD readings for 11 wells treated with one person’s serum diluted 1:25 (0.926) exceeded by more than a twofold the mean OD values for another (0.445). In each case, the coefficient of variation approximated 10%, and the difference was highly significant (p = 1 x 10^{-11}, unpaired t test). A significant difference was also observed at the higher dilution of 1:1000 (p = 0.003). Pretreatment of the wells with 20% foetal calf serum (FCS) for 2 hours failed to reduce the person to person variation, and the question arose as how to distinguish between positive or negative sera.

To overcome this problem I used eight sera from non-immunised persons as negative controls. They were serially diluted fivefold and corresponding dilutions of test and control sera were included on each plate. The OD reading above which a dilution of test serum was considered to be antibody positive was initially set at twice the mean of the OD readings of the negative controls. This discriminated against low titres of antibody when sera positive or negative for neutralising antibody were assayed, but closer agreement occurred when the mean OD value of negative controls plus 2.5 times the standard deviation was used instead.

During the early developmental stage it became evident that certain pre-vaccination sera that were negative by the MNT were giving higher OD readings than the negative controls, especially at low serum dilutions. Was the panel of eight negative sera unrepresentative of the population at large, or was there another explanation? Sera are heat-inactivated at 56°C for 30 minutes to remove non-specific inhibitors before titration for rabies neutralising antibody (Atanasiu, 1973). To ascertain whether this had any effect on non-specific binding I compared the OD readings of heat-inactivated and untreated sera from 17 non-immune subjects on antigen-coated plates. Interestingly the OD values of the heat-
inactivated sera (mean 0.8298; range 0.536 - 1.269) were significantly higher (p = 0.000009; paired t-test) than the OD values of the untreated control sera (mean 0.5158; range 0.366 - 0.831).

Rather than take the titre of antibody as the highest dilution giving an OD value greater than the mean plus 2.5 standard deviations of the mean of OD values of appropriate negative controls, it was felt that a more precise estimate of titre could be obtained if the OD values of the preceding and following dilutions were also taken into account. The idea of feeding OD values of test and control sera into a programmable calculator (or computer) and to calculate the titre mathematically was discussed with Doug Altman, MRC Division of Computing and Statistics, Northwick Park, who devised mathematical formulae to calculate the titres and compare the results with those obtained from scattergrams. We found that the calculated curve of best fit was drawn towards the high OD values (corresponding to the lower serum dilutions) and the resulting titres were consistently lower than those plotted by hand when OD results of four serial fivefold dilutions of test and control sera were used in the calculation. However, when we used formulae involving three OD readings for test and control sera, the curve-plot passed through all three points for OD values of test sera, and the calculated titres agreed closely with those provided by the scattergrams. Accordingly the method of calculation employed in the following studies involves:

(a) the inclusion of 8 negative control sera serially diluted fivefold on each plate,

and

(b) a calculator programmed to determine the intercept (x) between the quadratic curve fitted to the OD values of test sera, and the straight line fitted to the mean plus 2.5 standard deviations of the mean of OD values of controls. [The programme was kindly written by Dr R. Cormack,
3. Reproducibility of the ELISA test

The reproducibility of the assay was tested using a laboratory standard of pooled human serum with a rabies neutralising antibody titre of 1:1,000. Eight replicate titrations of the standard were made in two plates on five occasions. Using dilutions in the range 1:500 to 1:62,500, results were obtained for 78 of 80 titrations. Analysis revealed that 71 of 78 (91%) titres showed less than a twofold difference from the geometric mean (1:15,411), and no titre differed by more than a fourfold. For individual plates, the ratio of the highest to lowest titre ranged between 1.4 and 3.4 (mean = 2.4; SD 0.8); significant variation (p < 0.05) between duplicate plates was observed on one of five occasions with a 1.9-fold difference in mean titre. When coded sera were retitrated on separate occasions, 42 of 53 (79%) showed less than a twofold difference from the line of identity, and 96% differed by less than fourfold.

4. Comparison of ELISA and MNT

Sixty-nine sera were titrated once by MNT and on one or more occasions by ELISA; of the sera either positive or negative by both tests, there was complete agreement in 68 (98.5%). One sample that was positive by MNT, but negative by ELISA, was obtained seven days after vaccination when the majority, if not all, of the neutralizing antibody would be expected to be IgM. To quantify the relation between MNT and ELISA more precisely, the line of linear regression missing the origin (Snedecor & Cochran, 1967) and the correlation coefficient were determined. Analysis shows a log-log relationship between the results of the MNT and ELISA which is expressed by the formula:

$$\log_{10} \text{MNT} = 0.676 \log_{10} \text{ELISA} + 0.388$$
The formula shows that for relatively low concentrations of antibody (< 1:50) the titres measured by ELISA and MNT were numerically similar. However, with increasing concentrations of antibody, the titres measured by ELISA became increasingly greater than those measured by the MNT. The closeness of the data to the fitted line, however, is indicated by a correlation coefficient of 0.92.

5. Discussion

The data (Nicholson & Prestage, 1982) demonstrate that the ELISA test and method of antibody measurement together result in a rapid and highly reproducible assay suitable for use in serological studies of rabies.

Irregular non-specific reactions by sera from unimmunised subjects was initially a problem not resolved by pretreatment of plates with 20% foetal calf serum. This difficulty was overcome by including a number of non-immune sera in each test and arbitrarily choosing the cut-off point between antibody positive and negative sera as the mean + 2.5 standard deviations of the mean of the OD values of negative controls. Although this prevented the assay from being more sensitive than MNT with low titres of antibody, it reduced the likelihood of false-positive reactions to ~1:170. Moreover, absorbence values of test and control sera are assayed over a range of dilutions, which provides a more precise estimate of titres than any other method.

The rabies antigen used in the tests was prepared in bulk using MRC-5 human diploid cells and concentration and purification facilities usually employed for the production of human vaccines. Electron microscopy [kindly carried out by Ms Heather Davies, Clinical Research Centre, Harrow] showed it to be free of cellular material which may result in non-specific reactions. A control antigen, consisting of centrifuged WI-38 cell supernatants, was used during preliminary studies to exclude the possibility of false-positive reactions. Using HRIG and sera from a hyperimmunized individual, there was no evidence that recipients of HDCSV-WV developed antibodies to human diploid cell antigens similar to egg specific
antibody, which may develop in animals or humans given killed egg-grown influenza vaccine (Harboe et al, 1961).

Investigations reported by Wiktor et al (1973a) indicate that antibodies specific for the virus glycoprotein are responsible for neutralisation. It was originally considered that the whole virus particles with intact glycoprotein used in this ELISA should measure neutralising antibody mostly, and indeed the close agreement found between the MNT and ELISA supports this supposition. However, data from later postexposure treatment studies (See Chapter 10) suggest that the ELISA measures antibodies other than those directed against the glycoprotein and that such antibodies are present at high titre following NTV. Interestingly Dietzschold et al (1987) have shown that the viral ribonucleoprotein is involved in protection and acts synergistically with the surface glycoprotein, so it may be more appropriate to measure antibodies against both the ribonucleoprotein and glycoprotein rather than those just against the glycoprotein.

Of notable value is the ability of ELISA to detect antibodies of the IgG class. It is well known that IgM antibody is the initial humoral response to infection or immunisation, and that in infections with viraemia IgM is especially important. However, neither passively transferred nor actively induced IgM neutralising antibody protect mice infected experimentally (Turner, 1978). Thus IgG antirabies antibody detected by ELISA may be of greater importance than the early antibody response to infection or immunisation measured by neutralization tests.

The great advantage of the ELISA is that the antigen is inactivated and can be used safely in routine diagnostic laboratories. Moreover, many samples can be titrated quickly with relatively little technical expertise. These factors, together with its high degree of reproducibility, suggest that the ELISA will become an extremely useful technique for the sero-diagnosis of rabies and for measurement of response to vaccination. Results of its application in clinical trials in the UK, Pakistan, and Thailand are presented in the following Chapter.
1. Introduction

For more than 30 years the World Health Organisation has recommended vaccination of persons at risk of exposure to rabies (WHO, 1957). Both neural and avian tissue vaccines are unsatisfactory for this purpose either because they are poorly antigenic and require multiple injections or because they are associated with unacceptable clinical reactions (Miller & Nathanson, 1977; Turner, 1977; Crick & Brown, 1976). Human diploid cell strain rabies vaccine is produced from cultures of rabies virus infected human embryonic lung fibroblasts and is free from encephalitogenic factors and avian proteins; by 1975, HDCSV was known to reliably induce high titres of antibody without serious adverse reactions.

With only few exceptions, rabies is a problem of impoverished areas of the world where the annual per capita sum for health care is often considerably less than the cost of a single 1-ml dose of HDCSV. Consequently HDCSV has been a scarce commodity in the Third World, particularly to those most at risk. The following investigations were prompted by the need for an effective yet inexpensive method of treatment suitable for use in the Third World. In 1974, l'Institut Merieux kindly donated a quantity of HDCSV-WV to the MRC, and, in an attempt to find simple, effective and economic regimens for both pre- and postexposure prophylaxis, an assessment was made of the antigenicity and acceptability of vaccine by different routes of administration. In a preliminary communication, Aoki et al (1975) reported that the vaccine was well tolerated by the intramuscular (IM) and intradermal (ID) routes and that the antibody response to primary vaccination by
either route was excellent. In Study 1, I report the three-year results of pre-exposure vaccination of 194 volunteers with one, two, or three primary injections, and a booster dose at 6, 12, or 24 months.

By summer 1975 the MRC studies had already demonstrated that high titres of neutralising antibody are reliably produced when only one-tenth of the recommended 1.0ml dose is given intradermally. Since it was considered that both active and passive immunization should be given for severe rabies exposure and that human rabies immune globulin (HRIG) at a dose of 20 IU/kg was optimum, I immunised and plasmapheresed 16 volunteers and together with Dr George Turner compared the effects of HRIG (20 IU/kg) on the response to HDCSV-WV, administered by the IM and more economic ID route. The results are presented in Study 2.

The first two studies provide information concerning the clinical and humoral response to antirabies vaccination. Virus neutralizing antibody has long been considered to be the key to successful rabies prophylaxis, both before and after virus exposure (Habel, 1945; Kowrowski et al, 1950; Habel & Kowrowski, 1955; Winkler et al, 1969; Crick, 1973), and inadequate antibody responses to neurovaccine and duck embryo vaccine have generally been held responsible for occasional treatment failures. The absolute importance of virus neutralizing antibody has been questioned, however, by several observations. Sikes et al, 1971a & 1971b, noted that immunization prior to exposure occasionally fails to protect animals infected experimentally, despite the presence of circulating antibody at the time of virus challenge. In addition, many workers have found that passively administered antibody may prolong survival but have little influence on mortality (Sikes et al, 1971a; Yen, 1942; Kowrowski & Black, 1954; Baer & Cleary, 1972). Moreover the extremely high titres of neutralizing antibody present in the cerebrospinal fluid and blood of humans late in the disease has no apparent influence on the inevitably fatal outcome (Anderson et al, 1984). During the mid 1970's little was known of the roles of T-lymphocyte mediated, antibody-dependant cellular
(K-cell) immune responses, mononuclear phagocytes, or interferon, either in the immuno-pathogenesis of rabies or protection against it. Accordingly Studies 3 - 8 were undertaken to establish whether interferon-induction or cell-mediated responses occurred regularly after immunization with HDCSV.

Opponents to ID administration of rabies vaccine believe that it is technically difficult, especially in the elderly and very young. To determine whether a busy immunisation centre could achieve similar results to those obtained in the MRC studies, I obtained a number of coded sera from the British Airways Immunisation Centre for titration of rabies antibody by ELISA (Study 9), and also examined the possibility that a jet injector could simplify ID vaccination, yet give comparable results to those obtained by needle and syringe (Study 8).

The encouraging results obtained in the above studies led to a postexposure protection study in rabbits using a multisite intradermal schedule of immunisation (Study 10). This was followed by studies of the ID route of immunisation and cold chain in Pakistan (Study 11), and subsequently to simulated and actual postexposure trials of multisite HDCSV in Thailand.

The volunteer studies were all approved by the Northwick Park Hospital Ethical Committee and were undertaken with whole virion HDCSV that had been inactivated with β-propiolactone.

2. **Study 1 - A trial of pre-exposure prophylaxis with HDCSV**

Volunteers were allocated to receive one, two, or three doses of vaccine by the IM route in 1.0ml volumes, or by the ID route in 0.1ml volumes on days 0, 28, and 56. Serological results are available from 64 females aged 14 - 61 years (mean 30 years), and 130 males aged 16 to 68 years (mean 36 years); none had previously been immunized against rabies. A booster dose of vaccine was given at 6, 12, or 24 months by the ID (0.1ml) or deep subcutaneous (1.0ml) routes to randomly selected members of each group (Table 10.1).
Table 10.1 Population in Study 1 who were available for follow-up.

<table>
<thead>
<tr>
<th>Primary vaccination</th>
<th>No booster</th>
<th>6 Months:</th>
<th>12 Months:</th>
<th>24 Months:</th>
<th>Total</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>ID</td>
<td>SC</td>
<td>ID</td>
<td>SC</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ID</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>IM</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Day 0, 28:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ID</td>
<td>3</td>
<td>5</td>
<td>3</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>IM</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Day 0, 28, 56:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ID</td>
<td>7</td>
<td></td>
<td>7</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>IM</td>
<td>9</td>
<td></td>
<td>11</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
<td>18</td>
<td>34</td>
<td>31</td>
<td>29</td>
</tr>
</tbody>
</table>

The batches used had antigenic values of 1.7 and 5.9 (Lot No. S0203) and 1.6, 3.9, and 1.1 (Lot No. S0322) when tested for potency by the NIH method (Seligman, 1973) at different periods. Vaccine was stored at 4°C and reconstituted with pyrogen-free distilled water immediately before inoculation into the deltoid region of the left arm. The antibody responses to these regimens were compared on days 0, and at 1, 2, 3, 6, 12, 24, and 36 months, and one month after the booster dose. All volunteers were asked to complete a symptom questionnaire for 10 days after each inoculation, and the injection site was examined after 48 hours.
3. **Study 1 - Clinical reactions to vaccination**

HDCSV-WV was well tolerated, adverse reactions being no more severe than those found with other vaccines in common use. Generalized pruritus, rash, breathlessness, or wheezing developed in five persons. In three, the respiratory symptoms were clearly related to an upper respiratory tract infection, and in one other, a papular non-pruritic rash lasting for longer than 10 days was not considered to be a complication to vaccination. However, one 61-year old male with a history of penicillin hypersensitivity developed breathlessness and wheezing shortly after his second and third primary ID inoculations. Two years later, upon receiving a subcutaneous booster, he developed a generalised urticarial rash which responded to treatment with anti-histamines. The incidence and nature of clinical reactions are described in detail below:

*Reactions following primary intradermal vaccination:* 204 symptom questionnaires were completed after the 216 1st, 2nd, and 3rd 'primary' ID inoculations giving a 94.4% response rate. Chi-square analysis revealed no significant difference between the number of subjects who were symptom-free after each injection (Table 10.2).

**Table 10.2 Study 1; incidence of adverse reactions following primary ID vaccination on days 0, 28, and 56.**

<table>
<thead>
<tr>
<th></th>
<th>1st injection</th>
<th>2nd injection</th>
<th>3rd injection</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 28</td>
<td>Day 56</td>
<td></td>
</tr>
<tr>
<td>No. vaccinated</td>
<td>105</td>
<td>72</td>
<td>39</td>
<td>216</td>
</tr>
<tr>
<td>No. completing the questionnaire</td>
<td>105</td>
<td>61</td>
<td>38</td>
<td>204</td>
</tr>
<tr>
<td>No. with symptoms</td>
<td>74</td>
<td>40</td>
<td>26</td>
<td>140</td>
</tr>
<tr>
<td>% of total having symptoms</td>
<td>70.5</td>
<td>65.6</td>
<td>68.4</td>
<td>69</td>
</tr>
</tbody>
</table>
The nature of reactions and their duration were also similar after each of the three inoculations. The combined data for symptoms occurring within 10 days of 204 primary intradermal injections is shown in Table 10.3. Approximately 50% of vaccinees reported local erythema, 20% local swelling, 10% pruritus, and less than 5% complained of pain and other local reactions. There was no apparent relationship between systemic complaints and vaccination.

Table 10.3 Study 1; Symptoms occurring within 10 days of 204 intradermal injections given for primary immunisation.

<table>
<thead>
<tr>
<th>Symptoms:</th>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
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<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Local:</td>
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<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Redness</td>
<td>49</td>
<td>50.5</td>
<td>46</td>
<td>45</td>
<td>45</td>
<td>32</td>
<td>30</td>
<td>28</td>
<td>27</td>
<td>25.5</td>
</tr>
<tr>
<td>Swelling</td>
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<td>14</td>
<td>10</td>
<td>9</td>
<td>7</td>
<td>6</td>
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<td>5</td>
</tr>
<tr>
<td>Itching</td>
<td>7</td>
<td>11</td>
<td>11</td>
<td>8</td>
<td>7</td>
<td>6</td>
<td>3</td>
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</table>
An objective assessment of reactions was provided by a limited clinical examination 48 hours after 189 injections. Chi-squared analysis again revealed no significant differences between the incidence of local erythema, tenderness, induration, heat, regional adenopathy, or absence of signs after the 1st, 2nd, or 3rd inoculations. Altogether 89% of injections produced erythema and 65% induration, with mean surface areas of 1.04 and 0.4 cm² respectively; there was no significant difference between the diameters of erythema or induration after each inoculation (paired and unpaired t-tests). Local tenderness, heat, and regional adenopathy occurred after 11%, 15% and 7% of primary ID injection respectively (Table 10.4).

Table 10.4 Study 1; Clinical signs 48 hours after primary intradermal injection.

<table>
<thead>
<tr>
<th>Signs</th>
<th>Clinical signs after each injection:</th>
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<td>Third</td>
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<td>Erythema:</td>
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<tr>
<td>proportion (%)</td>
<td>87/100 (87)</td>
<td>52/57 (93)</td>
<td>29/32 (94)</td>
<td>168/189 (89)</td>
</tr>
<tr>
<td>diameter, mean</td>
<td>1.26 ± 0.96</td>
<td>1.16 ± 0.79</td>
<td>0.88 ± 0.49</td>
<td>1.15 ± 0.84</td>
</tr>
<tr>
<td>± s.d. (cm)</td>
<td>6.5</td>
<td>3.6</td>
<td>2.2</td>
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</tr>
<tr>
<td>max. diameter</td>
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<td></td>
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<td>Tenderness:</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>proportion (%)</td>
<td>10/100 (10)</td>
<td>7/57 (14)</td>
<td>4/32 (12.5)</td>
<td>21/189 (11)</td>
</tr>
<tr>
<td>Induration:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>proportion (%)</td>
<td>61/100 (61)</td>
<td>38/57 (67)</td>
<td>23/32 (72)</td>
<td>122/189 (65)</td>
</tr>
<tr>
<td>diameter, mean</td>
<td>0.69 ± 0.54</td>
<td>0.78 ± 0.5</td>
<td>0.64 ± 0.39</td>
<td>0.71 ± 0.5</td>
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<tr>
<td>± s.d. (cm)</td>
<td>3.0</td>
<td>2.5</td>
<td>2.0</td>
<td>3.0</td>
</tr>
<tr>
<td>max. diameter</td>
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<tr>
<td>Adenopathy:</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>proportion (%)</td>
<td>7/100 (7)</td>
<td>4/57 (7)</td>
<td>3/32 (9)</td>
<td>14/189 (7)</td>
</tr>
<tr>
<td>No signs:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>proportion (%)</td>
<td>9/100 (9)</td>
<td>6/57 (11)</td>
<td>2/32 (6)</td>
<td>17/189 (9)</td>
</tr>
</tbody>
</table>
Reactions following intradermal boosters: The possibility that repeated ID injections would cause hypersensitivity was assessed further after boosters. 114 symptom questionnaires were completed after revaccination (Table 10.5). Overall, there was a greater frequency of reactions to ID and SC boosters following primary ID vaccination than after primary IM injections (Chi-square test, .02 < p < .05). However, analysis of the subgroups given ID boosters after primary ID or IM vaccination, or SC boosters after primary ID or IM vaccination, failed to show any statistically significant differences.

Table 10.5 Study 1; Incidence of reactions after booster injections by the SC and ID routes.

<table>
<thead>
<tr>
<th>Primary IM regimen</th>
<th>SC booster</th>
<th>ID booster</th>
<th>SC booster</th>
<th>ID booster</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. vaccinated</td>
<td>29</td>
<td>18</td>
<td>35</td>
<td>32</td>
</tr>
<tr>
<td>No. with symptoms</td>
<td>14</td>
<td>7</td>
<td>23</td>
<td>21</td>
</tr>
<tr>
<td>% with symptoms</td>
<td>48</td>
<td>39</td>
<td>66</td>
<td>66</td>
</tr>
</tbody>
</table>

p value for comparison of symptoms occurring after ID boosters (columns 2 and 4) = .12; p value for symptoms after SC boosters (columns 1 and 3) = .25; for columns 1 and 2 combined versus 3 and 4 combined = .02 < p < .05.

Limited clinical examination at 48 hours showed that neither the incidence of erythema, tenderness, induration, heat, regional adenopathy and presence of signs (Table 10.6), nor the areas of erythema and induration, were significantly different for boosters after primary ID or IM regimens (Chi square and unpaired t-tests). Moreover, ID boosters following primary ID vaccination produced fewer symptoms and signs and for shorter periods than did primary immunisation by the ID route (Tables 10.3 & 10.7, Figure 10.1).
Table 10.6  Study 1; Signs 48 hours after ID and SC boosters (following primary (1°) IM and ID regimens).

<table>
<thead>
<tr>
<th>Booster</th>
<th>Sign</th>
<th>1° IM regimen</th>
<th>1° ID regimen</th>
<th>p-value IM vs ID</th>
</tr>
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<tbody>
<tr>
<td>0.1 ml ID</td>
<td>erythema</td>
<td>11/18</td>
<td>17/29</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>tenderness</td>
<td>1/18</td>
<td>2/29</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>induration</td>
<td>8/18</td>
<td>17/29</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>heat</td>
<td>2/18</td>
<td>4/29</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>adenopathy</td>
<td>0/18</td>
<td>0/29</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>no signs</td>
<td>5/18</td>
<td>10/29</td>
<td>ns</td>
</tr>
</tbody>
</table>

| 1.0 ml SC | erythema  | 8/26          | 8/27          | ns              |
|           | tenderness| 7/26          | 7/27          | ns              |
|           | induration| 8/26          | 7/27          | ns              |
|           | heat      | 3/26          | 4/27          | ns              |
|           | adenopathy| 2/26          | 1/27          | ns              |
|           | no signs  | 12/26         | 13/27         | ns              |
Table 10.7  Study 1; Symptoms occurring within 10 days of 50 intradermal boosters - 32 after 1° ID vaccination, 18 after 1° IM vaccination.

<table>
<thead>
<tr>
<th>Symptoms:</th>
<th>Percentage of injections followed by symptoms on days:</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4 5 6 7 8 9 10</td>
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<tr>
<td>Local (ID booster after 1° ID vaccination):</td>
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</tr>
<tr>
<td>Redness</td>
<td>44 34 22 19 9 6 6 6 6 6</td>
</tr>
<tr>
<td>Swelling</td>
<td>19 16 6 3 3 3 3 3 3 3</td>
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<td>Itching</td>
<td>12.5 9 9 6 - - - - - - -</td>
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<tr>
<td>Pain</td>
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<tr>
<td>Burning</td>
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<tr>
<td>Stinging</td>
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</tr>
<tr>
<td>Rash</td>
<td>- - - - - - - - - - - -</td>
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<tr>
<td>Local (ID booster after 1° IM vaccination):</td>
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<tr>
<td>Redness</td>
<td>28 17 17 5 5 5 5 5 5 5</td>
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<td>Systemic (ID booster after 1° IM and ID injections combined):</td>
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<td>- 2 2 - - - - - - - - - - - - - - - - - - - - - - -</td>
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Fig. 10.1 Predominant symptoms occurring within 10 days of primary ID vaccination (○) and an ID booster following primary ID vaccination (●).
Reactions following primary intramuscular vaccination: 172 symptom questionnaires were completed after 193 1st, 2nd, and 3rd 'primary' IM inoculations giving an 89.1% response rate. Symptoms were reported less frequently than after primary ID vaccination (Tables 10.3 & 10.8) with local pain and headache being the only symptoms recorded on more than 5% of occasions (16% and 6% respectively on day 1). The remaining symptoms occurred infrequently and bore no clear relation to vaccination.

Table 10.8 Study 1; Symptoms occurring within 10 days of 172 intramuscular injections given for primary immunisation.

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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Wheezing</td>
<td>-</td>
<td>0.5</td>
<td>0.5</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Naus/Vom</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Abd. pain</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>0.5</td>
<td>2</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
</tr>
</tbody>
</table>
165 injection sites were examined at 48 hours. There was a non-significant trend for signs to occur less frequently with ensuing inoculations, and overall, only 23% of injections caused local erythema, tenderness, induration, heat, or regional adenopathy (Table 10.9).

Table 10.9 Study 1; Clinical signs 48 hours after primary intramuscular injection.

<table>
<thead>
<tr>
<th>Signs</th>
<th>First</th>
<th>Second</th>
<th>Third</th>
<th>All three</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythema:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>proportion (%)</td>
<td>6/80 (7.5)</td>
<td>5/52 (10)</td>
<td>2/33 (94)</td>
<td>13/165 (8)</td>
</tr>
<tr>
<td>diameter, mean ± s.d. (cm)</td>
<td>0.5 ± 0.36</td>
<td>0.83 ± 0.69</td>
<td>0.9 ± 0.14</td>
<td>0.67 ± 0.47</td>
</tr>
<tr>
<td>max. diameter</td>
<td>1.0</td>
<td>1.8</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Tenderness:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>proportion (%)</td>
<td>5/80 (6)</td>
<td>3/52 (6)</td>
<td>0/33 (0)</td>
<td>8/165 (5)</td>
</tr>
<tr>
<td>Induration:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>proportion (%)</td>
<td>6/80 (7.5)</td>
<td>4/52 (8)</td>
<td>1/33 (3)</td>
<td>11/165 (7)</td>
</tr>
<tr>
<td>diameter, mean ± s.d. (cm)</td>
<td>0.23 ± 0.29</td>
<td>0.2 ± 0.1</td>
<td>0.5</td>
<td>0.25 ± 0.24</td>
</tr>
<tr>
<td>max. diameter</td>
<td>0.8</td>
<td>0.3</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Adenopathy:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>proportion (%)</td>
<td>1/80 (1)</td>
<td>1/52 (2)</td>
<td>0/33 (0)</td>
<td>2/165 (1)</td>
</tr>
<tr>
<td>No signs:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>proportion (%)</td>
<td>58/80 (72.5)</td>
<td>40/52 (77)</td>
<td>29/33 (88)</td>
<td>127/165 (77)</td>
</tr>
</tbody>
</table>

Reactions following subcutaneous boosters: 64 persons completed symptom questionnaires after SC boosters. Local symptoms were generally intermediate in frequency between those occurring after ID and IM vaccination (Table 10.10; See also Tables 10.3 and 10.8).
Table 10.10  Study 1: Symptoms occurring within 10 days of 64 subcutaneous boosters - 35 after $1^\circ$ ID vaccination, 29 after $1^\circ$ IM vaccination.

<table>
<thead>
<tr>
<th>Symptoms:</th>
<th>Percentage of injections followed by symptoms on days:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Local (SC booster after $1^\circ$ ID vaccination):</td>
<td></td>
</tr>
<tr>
<td>Redness</td>
<td>34</td>
</tr>
<tr>
<td>Swelling</td>
<td>17</td>
</tr>
<tr>
<td>Itching</td>
<td>3</td>
</tr>
<tr>
<td>Pain</td>
<td>8</td>
</tr>
<tr>
<td>Burning</td>
<td>8</td>
</tr>
<tr>
<td>Stinging</td>
<td>3</td>
</tr>
<tr>
<td>Rash</td>
<td>-</td>
</tr>
<tr>
<td>Local (SC booster after $1^\circ$ IM vaccination):</td>
<td></td>
</tr>
<tr>
<td>Redness</td>
<td>31</td>
</tr>
<tr>
<td>Swelling</td>
<td>14</td>
</tr>
<tr>
<td>Itching</td>
<td>3</td>
</tr>
<tr>
<td>Pain</td>
<td>17</td>
</tr>
<tr>
<td>Burning</td>
<td>-</td>
</tr>
<tr>
<td>Stinging</td>
<td>-</td>
</tr>
<tr>
<td>Rash</td>
<td>-</td>
</tr>
<tr>
<td>Systemic (SC booster after $1^\circ$ IM and ID injections combined):</td>
<td></td>
</tr>
<tr>
<td>Malaise</td>
<td>2</td>
</tr>
<tr>
<td>Aching</td>
<td>3</td>
</tr>
<tr>
<td>Headache</td>
<td>6</td>
</tr>
<tr>
<td>Pruritus</td>
<td>3</td>
</tr>
<tr>
<td>Fever</td>
<td>-</td>
</tr>
<tr>
<td>Chills</td>
<td>2</td>
</tr>
<tr>
<td>Dizziness</td>
<td>-</td>
</tr>
<tr>
<td>Gen. rash</td>
<td>2</td>
</tr>
<tr>
<td>Dyspnoea</td>
<td>-</td>
</tr>
<tr>
<td>Wheezing</td>
<td>-</td>
</tr>
<tr>
<td>Naus/Vom</td>
<td>-</td>
</tr>
<tr>
<td>Abd. pain</td>
<td>-</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>-</td>
</tr>
</tbody>
</table>
53 injection sites were examined 48 hours after vaccination. As with ID boosters, the incidence of erythema, tenderness, induration, heat, regional adenopathy, and absence of signs were virtually identical after primary IM and ID regimens (See Table 10.6). Table 10.11 shows the signs that occurred after ID and SC boosters with the subgroups previously vaccinated by the IM and ID routes being combined.

Table 10.11  Study 1; Clinical signs 48 hours after ID and SC boosters.

<table>
<thead>
<tr>
<th>Signs</th>
<th>Clinical signs after boosting:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ID booster</td>
</tr>
<tr>
<td>Erythema:</td>
<td></td>
</tr>
<tr>
<td>proportion (%)</td>
<td>28/47 (60)</td>
</tr>
<tr>
<td>diameter, mean</td>
<td>1.37 ± 0.95</td>
</tr>
<tr>
<td></td>
<td>ID booster</td>
</tr>
<tr>
<td>Tenderness:</td>
<td>3/47 (6)</td>
</tr>
<tr>
<td>Induration:</td>
<td>25/47 (53)</td>
</tr>
<tr>
<td>proportion (%)</td>
<td>0.37 ± 0.26</td>
</tr>
<tr>
<td>adenopathy:</td>
<td>0/47 (0)</td>
</tr>
<tr>
<td>proportion (%)</td>
<td>15/47 (32)</td>
</tr>
<tr>
<td>No signs:</td>
<td></td>
</tr>
</tbody>
</table>

Summary of symptoms and signs: Tables 10.12 and 10.13 summarise the predominant symptoms and clinical signs after primary and booster injections. The IM route was evidently tolerated best, 65% of injections being symptom-free and 77% producing no clinical signs; however, this route caused pain in 16% of cases. In contrast, the majority of ID injections were accompanied by small areas of
Table 10.12 Study 1; Summary of predominant symptoms after primary and booster injections.

<table>
<thead>
<tr>
<th></th>
<th>Primary vaccination</th>
<th>Booster vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IM</td>
<td>ID</td>
</tr>
<tr>
<td>No. of injections</td>
<td>172</td>
<td>204</td>
</tr>
<tr>
<td>% without symptoms</td>
<td>65</td>
<td>31</td>
</tr>
<tr>
<td>% with erythema</td>
<td>4</td>
<td>50.5</td>
</tr>
<tr>
<td>% with swelling</td>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td>% with itching</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>% with pain</td>
<td>16</td>
<td>3</td>
</tr>
<tr>
<td>% with headache</td>
<td>6</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 10.13 Study 1; Summary of reactions to primary and booster injections by the IM, ID, and SC routes.

<table>
<thead>
<tr>
<th></th>
<th>Primary vaccination</th>
<th>Booster vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IM</td>
<td>ID</td>
</tr>
<tr>
<td>No. of injections</td>
<td>165</td>
<td>189</td>
</tr>
<tr>
<td>% without signs</td>
<td>77</td>
<td>9</td>
</tr>
<tr>
<td>% with erythema</td>
<td>8</td>
<td>89</td>
</tr>
<tr>
<td>erythema, mean</td>
<td>0.67</td>
<td>1.15</td>
</tr>
<tr>
<td>diameter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% with tenderness</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>% with induration</td>
<td>7</td>
<td>65</td>
</tr>
<tr>
<td>induration, mean</td>
<td>0.25</td>
<td>0.71</td>
</tr>
<tr>
<td>diameter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% with heat</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>% with adenopathy</td>
<td>8</td>
<td>7</td>
</tr>
</tbody>
</table>

erythema and induration, but little pain. The subcutaneous route produced symptoms and signs intermediate in frequency with those of the IM and ID routes. With the exception of headache which occurred in 6% of vaccinees after IM and SC vaccination, all systemic reactions occurred in less than 5% of occasions.
irrespective of the route of immunisation.

4. Study 1 - Antibody responsiveness: effect of age, sex, and vaccine batch

Before comparing the antibody responses to the various regimens, it was necessary to establish whether the two vaccine batches, Lot Numbers S0203 and S0322, were equipotent for man. Analysis by unpaired t-test showed that there were no significant differences between the antibody responses to the first injection with either batch of vaccine, either by the IM (GMT 2.8 versus 3.1, batches S0203 and S0322 respectively) or ID route (GMT 3.9 versus 2.7, batches S0203 and S0322 respectively). It was further established that the humoral response was unaffected by sex, consequently results for both males and females receiving either batch of vaccine were aggregated for statistical analysis.

The possible effect of age on antibody responsiveness to the first dose of vaccine was also studied. Sub-group analysis of day 28 antibody titres generally showed a tendency for decreased antibody responsiveness with increasing age which became statistically significant (Regression analysis, .025 < p ≥ .05) when all the data were combined (Figure 10.2). However, the proportion of people aged 50 and above who failed to develop titres of ≥ 0.5 IU/ml was no different to those who were younger. Moreover, analysis of day 56 titres showed that there was no significant effect of age on antibody responsiveness after two IM or ID injections. Review of the age distribution of the IM and ID groups showed that they were well matched, indicating that any differences in antibody titres were related to the regimen rather than age, sex, or vaccine batch.

Altogether 1383 sera were tested by Dr George Turner for virus neutralising antibodies by the mouse neutralization technique. 76 sera taken 36 months after immunization were titrated for antirabies IgG using ELISA. Antibody responses to the various regimens were compared by an unpaired t-test. Booster responses were
analyzed with respect to both the route and number of doses used in primary immunization and to the route and timing of the booster doses. For this purpose data from individual groups were pooled and compared both by titre and by the ratio of mean titre increase.

Fig. 10.2 Regression of age against the antibody titre (IU/ml) 28 days after the first ID or IM injection with batches S0203 and S0322.
Study 1 - Antibody response to primary immunization

The geometric mean titre (GMT) of antibody, the range of titres, the proportion of subjects without detectable antibody (<0.1 IU/ml) and the proportion with titres less than 0.5 IU/ml are shown in Table 10.14.

**Immunization on day 0 only.** Twentyeight days after immunization, neutralizing antibody was present (≥ 0.1 IU/ml) in 99 of 101 (98%) persons inoculated ID and in 83 of 86 (96.5%) persons inoculated IM. There was a higher GMT in the ID group (3.4 IU/ml) than in the IM group (2.8 IU/ml) but the difference was not statistically significant. The range of the antibody responses was wide (< 0.1 - 44 IU/ml); 72 and 78% of the results differed by less than fourfold from the GMT's for IM and ID groups respectively. The titres fell rapidly but were similar after both IM and ID immunization at each of the different time periods; by 6 months 28% of all vaccinees had titres of less than 0.5 IU/ml and 18% had no detectable antibody (<0.5 IU/ml).

**Immunization on days 0 and 28.** One hundred per cent of 127 vaccinees had titres of neutralizing antibody greater than 0.5 IU/ml 28 days after the second injection. The range remained wide (0.7 - 216 IU/ml), but the majority of titres (91 and 76%) again differed by less than fourfold from GMTs of 6.8 and 14.7 IU/ml for ID and IM immunization respectively. At 2, 6, and 12 months, the GMTs were 2.2 - 4.8 times higher after immunization by the IM route and the differences were statistically significant. The neutralizing antibody persisted for longer and at higher levels after two injections than after one (Table 10.14). However, at 6 months, 1 of 24 (4%) persons immunized ID no longer had detectable antibody; at 12 months, neutralizing antibody was absent from the sera of 2 of 20 (10%) persons inoculated ID and 1 of 15 (7%) who were inoculated IM.

**Immunization on days 0, 28 and 56.** 28 days after a third injection, the GMTs had increased from 6.8 to 10.4 IU/ml (1.5-fold), and from 14.7 to 18.7 IU/ml (1.3-fold) for ID and IM immunization respectively. The range of the
Table 10.14 Study 1; antibody titres after primary immunization with HDCS rabies vaccine.

<table>
<thead>
<tr>
<th>Immunization schedule</th>
<th>Neutralizing antibody (IU/ml) months after first dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>0.1 ml ID on day 0</td>
<td></td>
</tr>
<tr>
<td>'n'</td>
<td>101</td>
</tr>
<tr>
<td>GMT</td>
<td>3.4</td>
</tr>
<tr>
<td>Range</td>
<td>&lt; 0.1-32</td>
</tr>
<tr>
<td>% &lt; 0.1</td>
<td>2</td>
</tr>
<tr>
<td>% &lt; 0.5</td>
<td>4</td>
</tr>
<tr>
<td>1.0 ml IM on day 0</td>
<td></td>
</tr>
<tr>
<td>'n'</td>
<td>86</td>
</tr>
<tr>
<td>GMT</td>
<td>2.8</td>
</tr>
<tr>
<td>Range</td>
<td>&lt; 0.1-44</td>
</tr>
<tr>
<td>% &lt; 0.1</td>
<td>3.5</td>
</tr>
<tr>
<td>% &lt; 0.5</td>
<td>6</td>
</tr>
<tr>
<td>0.1 ml ID on days 0 &amp; 28</td>
<td></td>
</tr>
<tr>
<td>'n'</td>
<td>101</td>
</tr>
<tr>
<td>GMT</td>
<td>3.4</td>
</tr>
<tr>
<td>Range</td>
<td>&lt; 0.1-32</td>
</tr>
<tr>
<td>% &lt; 0.1</td>
<td>2</td>
</tr>
<tr>
<td>% &lt; 0.5</td>
<td>4</td>
</tr>
<tr>
<td>1.0 ml IM on days 0 &amp; 28</td>
<td></td>
</tr>
<tr>
<td>'n'</td>
<td>86</td>
</tr>
<tr>
<td>GMT</td>
<td>2.8</td>
</tr>
<tr>
<td>Range</td>
<td>&lt; 0.1-44</td>
</tr>
<tr>
<td>% &lt; 0.1</td>
<td>3.5</td>
</tr>
<tr>
<td>% &lt; 0.5</td>
<td>6</td>
</tr>
<tr>
<td>0.1 ml ID on days 0, 28 and 56</td>
<td></td>
</tr>
<tr>
<td>'n'</td>
<td>101</td>
</tr>
<tr>
<td>GMT</td>
<td>3.4</td>
</tr>
<tr>
<td>Range</td>
<td>&lt; 0.1-32</td>
</tr>
<tr>
<td>% &lt; 0.1</td>
<td>2</td>
</tr>
<tr>
<td>% &lt; 0.5</td>
<td>4</td>
</tr>
<tr>
<td>1.0 ml IM on days 0, 28 and 56</td>
<td></td>
</tr>
<tr>
<td>'n'</td>
<td>86</td>
</tr>
<tr>
<td>GMT</td>
<td>2.8</td>
</tr>
<tr>
<td>Range</td>
<td>&lt; 0.1-44</td>
</tr>
<tr>
<td>% &lt; 0.1</td>
<td>3.5</td>
</tr>
<tr>
<td>% &lt; 0.5</td>
<td>6</td>
</tr>
</tbody>
</table>

Note: 'n' = the number of sera tested at each time period; GMT, geometric mean titre <0.1, no detectable antibody.
antibody titres was again wide (0.5 - 216 IU/ml); 81% of titres after ID immunization, and 76% after IM immunization, differed by less than fourfold from the GMT. At 3, 6 and 12 months, the GMTs were 1.7 - 2.3 times higher after immunization by the IM route and the differences were statistically significant. At 24 months, 100% of 34 subjects still had neutralizing antibody. At 36 months, the sera of 5 persons vaccinated ID, and 7 of 9 vaccinated IM still had antirabies antibody as measured by ELISA.

6 Study 1 - Antibody response to booster immunization

Sera from a total of 150 persons were taken immediately before and 28 days after re-immunization at 6, 12, and 24 months. A single booster dose resulted in a substantial increase of virus neutralizing antibodies in most subjects; only 2 of 150 persons (1.3%) failed to develop titres greater than 1.0 IU/ml, and more than 95% had titres greater than 5.0 IU/ml. Analysis of the aggregated data shows that the route of administration of the primary regimen had no significant effect either upon the titre increase or the titres that were attained, although the antibody levels before re-immunization were significantly lower (~2-fold) in persons previously injected by the ID route (Table 10.15A). After one, two, or three primary doses, an inverse relationship existed between the pre-immunization titres and the increase in the mean titres which differed significantly from group to group; nevertheless the one dose regimen was as effective as two- or three-dose regimens when the titres after re-immunization were compared (Table 10.15B). Antibody titres measured before ID and SC booster injections were not significantly different, although they tended to be higher in the group subsequently inoculated subcutaneously. While the ratio of the titre increase was higher after re-immunization by the SC route the difference was not statistically significant. However, a comparison of the actual titres after re-immunization showed that they were significantly higher (~2-fold) after SC boosters (p = < 0.001) (Table 10.16A). Analysis revealed no significant differences
between the antibody titres of sera taken 28 days after booster injections at 6, 12, or 24 months; however, the ratio of increase was significantly lower in subjects boosted at 6 months (Table 10.16B) because the titre before boosting was higher.

Table 10.15 Study 1; Effect of route and dosage of primary immunization on the antibody response measured 28 days after a booster dose.

<table>
<thead>
<tr>
<th>GMT (IU/ml)</th>
<th>No. of Subjects</th>
<th>Before boost</th>
<th>After boost</th>
<th>Mean titre elevation</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(A) Route of primary immunisation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ID</td>
<td>81</td>
<td>0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.3</td>
<td>35.5</td>
<td>0.7-416</td>
</tr>
<tr>
<td>IM</td>
<td>69</td>
<td>1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.5</td>
<td>25.1</td>
<td>3.3-954</td>
</tr>
<tr>
<td>(B) No. of primary doses</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>48</td>
<td>0.4&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>44.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>105.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.7-954</td>
</tr>
<tr>
<td>2</td>
<td>46</td>
<td>1.4&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>49.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>36.6&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>2.4-389</td>
</tr>
<tr>
<td>3</td>
<td>56</td>
<td>2.7&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>23.8&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>8.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.7-316</td>
</tr>
</tbody>
</table>

Statistical significance of the difference between the pairs of figures suffixed is as follows: 'a' denotes $P < 0.05, > 0.01$; 'b' denotes $P = < 0.01, > 0.001$; 'c' denotes $P = < 0.001$.

Table 10.16 Study 1; Effect of route of administration and timing of booster doses on the antibody response measured 28 days after the booster doses.

<table>
<thead>
<tr>
<th>GMT (IU/ml)</th>
<th>No. of Subjects</th>
<th>Before boost</th>
<th>After boost</th>
<th>Mean titre elevation</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(A) Route of booster dose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ID</td>
<td>72</td>
<td>1.0</td>
<td>25.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>25.8</td>
<td>0.7-219</td>
</tr>
<tr>
<td>SC</td>
<td>78</td>
<td>1.5</td>
<td>51.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>34.9</td>
<td>3.3-954</td>
</tr>
<tr>
<td>(B) Timing of booster dose in months</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>48</td>
<td>1.6</td>
<td>28.1</td>
<td>17.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.4-954</td>
</tr>
<tr>
<td>12</td>
<td>51</td>
<td>0.9</td>
<td>43.7</td>
<td>46.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.4-416</td>
</tr>
<tr>
<td>24</td>
<td>51</td>
<td>1.2</td>
<td>38.8</td>
<td>32.2</td>
<td>7-158</td>
</tr>
</tbody>
</table>

Statistical significance of the difference between the pairs of figures suffixed is as follows: 'b' denotes $P = < 0.01, > 0.001$; 'c' denotes $P = < 0.001$.  

Study 1 - Rapidity of booster responses

Antibody assays on serial, serum samples taken after booster doses of vaccine showed that the responses were rapid. An upward trend in mean titre was apparent after 48 hours although one of the subjects appeared to be a slow responder. Four of the five individuals had statistically significant titre increases by 4 days (paired t test) and after 7-8 days very substantial increases had occurred in all subjects. The mean titre elevation at this time was approximately 23-fold but individual responses showed variations between 5 and 60 times their pre-boost levels. The assays also showed that peak antibody titres probably occurred between 8 and 16 days and declined between 16 and 28 days after the booster dose (Table 10.17).

Table 10.17 Study 1; The rapidity of the antibody response to booster doses of vaccine.

| Antibody response (IU/ml) at days after booster doses of vaccine |
|---------------------|-------|-------|-------|-------|-------|-------|-------|-------|
| Subject No. | 0 | 1 | 2 | 4 | 7 | 8 | 16 | 28 |
| 1          | 2.4 | - | 8.7 | 8.7 | - | 60 | 83 | 8.7 |
| 2          | 6.3 | - | 17 | 32 | - | 32 | 60 | 8.7 |
| 3          | 0.5 | 0.5 | - | 17 | 30 | - | 60 | 17 |
| 4          | 1.7 | 8.7 | 4.6 | 12 | - | - | 44 | 5.2 |
| 5          | 4.6 | 6.3 | 4.0 | 6.3 | - | 115 | 32 | 43 |
| GMT       | 2.3 | 3.0 | 7.4 | 13 | 51 | 53 | 12.4 |

significance: NS, .05-.1, .02-.05, .01-.02, <.001, .05-.1

GMT, geometric mean titres; NS, not statistically significant from pre-boost value.
8 Study 1 - Antibody titres at 36 months

At 36 months, the MNT and ELISA test detected antirabies antibody in all of 142 persons who had received one, two, or three primary doses and a booster. Analysis showed no statistically significant differences between the titres of neutralizing antibody of groups re-immunized after one or two primary inoculations by the IM or ID routes. However, as found earlier, the titres were significantly greater (~2-fold) when the booster injection was given SC.

9 Study 1 - Discussion

Local reactions occurred frequently with the intradermal regimens but were clinically insignificant. Generalized reactions such as malaise, headache, fever, and lymphadenopathy were reported infrequently, and with the exception of headache, which occurred in 6% of subjects after IM and SC injections, there was no consistent relationship between generalized symptoms and vaccination and the reaction rates were similar after ID and IM vaccinations. Symptoms were less frequent after ID boosters than after primary ID vaccination. Similarly, Anderson et al (1960) found that reactions to ID booster doses of duck embryo vaccine were consistently milder than those encountered during primary ID vaccination.

The only hypersensitivity reactions in this study occurred in an atopic individual after primary and booster doses of vaccine. Cox & Schneider (1976) observed local reactions in 7 of 14 subjects after ID primary and booster inoculations with Merieux HDCSV; 9-10 days after the booster, 2 of the 7 individuals complained of generalised pruritus, urticaria, and oedema of the face, legs, arms, and hips. By May 1984, over a period of 48 months, 108 allergic reactions mostly in association with boosters had been reported in the USA with reactions ranging from urticaria to anaphylaxis (11 per 10 000 vaccinees) (MMWR, 1984). Preliminary analysis of epidemiologic features of the illness revealed a male/female relative risk of 2.3. However, no significant associations...
were demonstrated between persons who reported presumed Type III hypersensitivity reactions and age, route of primary or booster immunization (IM or ID), timing of booster after primary immunization, history of other allergies, or history of previous immunization with rabies vaccines other than HDCSV. These reports of systemic allergic reactions included 9 cases of presumed Type I hypersensitivity (1: 10 000), 87 cases of presumed Type III delayed hypersensitivity (9: 10 000), and 12 cases of indeterminate type of allergic reactions (Winkler, 1985). Skin testing of 5 persons experiencing the reaction showed that all 5 reacted strongly to the Merieux HDCSV, but only one showed any reactivity to the more purified Behringwerke vaccine and that was very weak (Anon, JAMA, 1985). Radioallergosorbent testing (RAST) on sera from 4 patients who had also experienced allergic reactions showed that they all reacted positively to the Merieux HDCSV and to β-propiolactone-treated human serum albumin, but not to an HDCV not inactivated by β-propiolactone, to DEV inactivated by β-propiolactone, or to human serum albumin (Baer et al, 1985). It was also shown that β-propiolactone treated homologous serum albumin caused anaphylaxis in 70% of guinea-pigs. (Levenbrook et al, 1986). It was concluded that the β-propiolactone-treated human serum albumin component of the vaccine induced the allergic reactions seen clinically (Anon, JAMA, 1985).

No neurological reactions were observed during this (or subsequent studies). However, in a large follow-up study of reaction conducted in Britain between 1976 and 1983 and covering more than 40 000 doses for both pre- and postexposure immunization (Gardner, 1983), two neurological reactions were observed: one was transient in a person who experienced paraesthesiae and weakness of the right arm after the fourth dose of a post exposure course; the second had pain and weakness of the right arm with muscle wasting after a routine booster dose. Two other cases have been reported. Boe & Nyland (1980) describe one case of Guillain-Barré syndrome (GBS) in a 15 year old boy who received two ID doses of Merieux vaccine 30 days apart and who developed GBS 50 days after the first injection. Bernard et al
(1982a) reported an 11 year old boy with transient muscular leg weakness. Even if all four cases are accepted as being due to vaccination by HDCSV, the neuro-complication rate would be only ≤ 1: 250 000, similar to the values of most safe vaccines such as oral poliomyelitis, yellow fever, and tetanus toxoid.

Recommendations for pre-exposure immunization advise that a minimum antibody level of 0.5 IU/ml should be attained 4 weeks after the last inoculation (WHO Epidem Rec, 1978b). In the present investigation 96% of subjects given a single dose of 0.1 ml of vaccine ID, and 94% given 1.0 ml IM had titres equal to, or in excess of, this arbitrary level. However, the titres rapidly declined, and by 6 months 28% of vaccinees had titres less than 0.5 IU/ml and 19% had no detectable antibody (<0.1 IU/ML), clearly indicating that more than one dose is necessary.

Twenty eight days after a second injection by either the IM or ID routes, 100% of vaccinees had titres in excess of 0.5 IU/ml. These results confirmed the group's preliminary observations (Aoki et al, 1975; Nicholson et al, 1978) and are in agreement with those of studies in Germany (Cox & Schneider, 1976), France (Ajjan et al, 1978), and Belgium (Costy-Berger, 1978) where two doses of Merieux HDCS vaccine were given 28 days apart. Although the study reveals that the titres were significantly higher using the IM route, the proportion of vaccinees who were without antibody 6-24 months after immunization were similar whether the IM or ID routes were used and it is questionable whether the significantly higher titres which develop after IM immunization are clinically important. The results suggest that two doses of vaccine given 28 days apart by the ID (0.1 ml) or IM (1.0 ml) routes are adequate for pre-exposure prophylaxis where the risk of exposure is low. Such categories might include certain veterinary surgeons, workers in quarantine facilities, customs officials, and persons, especially children, living in or visiting countries where rabies is endemic. The results also suggest that with HDCSV-WV of adequate potency (antigenic value > 2.5) there is little justification for assaying the antibody responses of low risk subjects. The necessity for frequent
booster injections also appears doubtful.

A well monitored and solid immune response is probably more important for persons working with live rabies virus in research, diagnostic laboratories, and in vaccine production facilities where risks of accidental exposure may be higher. The data show that although antibody titres increased by only 1.3 to 1.5-fold for IM and ID immunization 28 days after a third dose, this latter injection reinforced the humoral response so that antibody was still present in 100% of vaccinees at 2 years and in 86% at 3 years. Nevertheless, a small proportion of vaccinees (5/69, 7%) only had titres in the range 0.5-1.0 IU/ml three months after their third injection. Thus, it is probably necessary to monitor antibody titres of individuals at high risk of exposure at 6-monthly intervals and give booster injections as required.

It is noteworthy that although the height of antibody response to the first vaccination decreased with age, the proportion of people aged 50 and above who failed to develop titres of ≥ 0.5 IU/ml was no different to those who were younger, moreover the titres were indistinguishable from those of younger people following a second dose. Hefti et al (1979) similarly found that the titres of persons 60 years of age and over are less than those of persons aged under 25, but that titres of < 0.5 IU/ml are no more frequent in the elderly than in the young.

A single booster generally induced a rapid and marked anamnestic response irrespective of the primary regimen or the timing and route of re-immunization or initial antibody titre. These data extended our preliminary observations (Nicholson et al, 1978) and have been confirmed by other workers (Rosanoff & Tint, 1979; Simona, et al, 1979; Fayaz et al, 1981). The rapidity of the booster doses is of some significance in previously immunised subjects who are subsequently exposed. The data indicate that in general, responses begin at 48 hours and are substantial 96 hours after a single dose. Peak values occur after 8-16 days, well within the average incubation period in human rabies. Only 2 of 150 persons (1.3%) failed to develop titres greater than 1.0 IU/ml, and more than 95% had titres greater than
5.0 IU/ml. In view of this (Nicholson et al, 1978; Turner et al, 1982) and similar data, the WHO Expert Committee on Rabies recommends that a person who, as a result of pre-exposure vaccination or postexposure treatment, has demonstrated an antibody response in the past, be given one booster dose when subsequently exposed to rabies. In very severe exposures (e.g., head bites) or when there is doubt about the potency of the immunization schedule used, the Committee suggests that additional booster doses of vaccine should be considered, e.g., on days 0, 3, and 7. The Committee also recommends that persons who have previously received full pre- or postexposure treatment with a vaccine of proven potency (e.g., HDCSV), but whose neutralizing antibody titre has not been determined, should receive 3 doses of a vaccine of proven potency on days 0, 3, and 7 after re-exposure (WHO, 1984).

10 Study 2 - Human rabies immune globulin (HRIG) and postexposure immunization

Study 1 shows that high titres of neutralising antibody are reliably produced when only one-tenth of the recommended dose is given intradermally. Since active and passive immunisation are both considered essential components of postexposure treatment, I compared the effects of HRIG on the titres evoked by HDCS vaccine administered by the IM and more economic ID routes. The high cost of commercially available HRIG limited its use in the trial and it was necessary to vaccinate and plasmapherese volunteers to provide material for the study.

Seventy healthy students and staff at the Royal Veterinary College, London, who had not previously received antirabies therapy, took part in the study. HDCSV-WV, Lot M1200, antigenic value 4.1, was donated by Institut Merieux, Lyon. HRIG, Lot 4912 (Cutter Laboratories), obtained from the Central PHLS Laboratory, Colindale, had a quoted potency of 150 IU/ml and gammaglobulin content of 16.5 mg%. HRIG Lot GR1 was obtained from the Blood Products Laboratory, Lister Institute and had a potency of 500 IU/1.2ml and gammaglobulin content of 10.5 mg%. HDCSV was given IM (1.0ml) or ID (0.1ml) on days 0, 3, 7, and 14, and HRIG
was given at the time of the first vaccine dose on day 0. The allocation of vaccine
and/or HRIG to the seven groups of volunteers is shown in Table 10.18.

Table 10.18 Study 2; Schedules of sero-vaccine therapy.

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccine* (days 0,3,7,14)</th>
<th>HRIG**</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>Cutter</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>Lister</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

* administered in the deltoid region of one arm only
** given IM in two divided doses into the gluteal muscles immediately after the vaccine.

Table 10.19 Study 2; Relation of dose of HRIG to mean weight, age, and sex of groups receiving vaccine and HRIG ± vaccine.

<table>
<thead>
<tr>
<th>HRIG</th>
<th>Weight</th>
<th>Age</th>
<th>Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>IU</td>
<td>ml</td>
<td>Kg</td>
<td>years</td>
</tr>
<tr>
<td>Vaccine only</td>
<td>-</td>
<td>-</td>
<td>65.7</td>
</tr>
<tr>
<td>Lister RIG</td>
<td>-</td>
<td>-</td>
<td>66.5</td>
</tr>
<tr>
<td>Cutter RIG</td>
<td>-</td>
<td>-</td>
<td>65.5</td>
</tr>
</tbody>
</table>

Blood samples were obtained immediately prior to vaccination and subsequently on
days 1, 2, 3, 7, 10, 14, 21, 30, 50, and 90. Dr Turner assayed antibody in all sera
from 5 members from each group. The comparability of the groups is shown in
Table 10.19. Each volunteer completed a symptom questionnaire for a period of 21
days and inoculation sites were examined throughout the study. All volunteers
tolerated sero-vaccination well and there were no untoward reactions.

11 Study 2 - Serological results

Antibody response to vaccination only: groups 4 and 7. Rabies neutralizing antibody was not found before day 7. On day 7 it was found in 3/5 of the IM group and 2/5 of the ID group. By day 10 all volunteers had antibody titres $\geq 0.5$ IU/ml; no significant differences were observed between the peak antibody titres obtained by either route between days 21 and 30 (standard error of the difference between means, 95% level of confidence) (Figure 10.3).

Vaccine and Lister immune globulin: groups 2 and 3. When 20 IU/kg of Lister HRIG was given with ID or IM therapy, there was no delay in the antibody response of the peak geometric mean titres (GMT) if compared with 'vaccine only' groups. However, the titres after IM vaccination and HRIG were significantly higher than after ID vaccination and HRIG (Figure 10.3).

Lister and Cutter HRIG: groups 1 and 5. Since active immunization produced no detectable neutralizing antibody response prior to day 7, a comparison was made of antibody detected during the first three days in all volunteers given Lister or Cutter HRIG (Table 10.20). 24 hours after passive immunization with Cutter HRIG, all volunteers had detectable antibody; barely half of those given Lister HRIG had antibody and it was of extremely low titre. No measurable circulating antibody was found throughout the period of the study in one of 15 volunteers given Lister HRIG. The peak titre of both groups was observed on day 2. Throughout the first three days the GMTs in the Cutter group were between 5 and 11 times greater than those in the Lister group. The antibody profiles are shown in Figure 10.4.

Vaccine and Cutter HRIG: group 6. Although the titres of neutralising antibody during the first three days after giving Cutter HRIG were higher than those after Lister HRIG, no suppression of the antibody response to 1.0 ml quantities of vaccine given by the IM route was observed (Figure 10.4).
Potency evaluation of Cutter HRIG. Because of the different antibody profiles which were observed following passive immunization with 20 IU/kg of Lister and Cutter HRIG, Dr GS Turner tested the potency of Cutter HRIG. Five months after the expiry date its titre was above the expected titre range; on re-testing, the titre was 380 IU/ml; this exceeded the quoted potency 2.5-fold.

Table 10.20 Study 2; Comparison of GMTs following immunization with 20IU/kg Lister and Cutter HRIG.

<table>
<thead>
<tr>
<th>HRIG</th>
<th>GMT IU/ml</th>
<th>% with antibody</th>
<th>GMT IU/ml</th>
<th>% with antibody</th>
<th>GMT IU/ml</th>
<th>% with antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lister</td>
<td>0.06</td>
<td>47</td>
<td>0.18</td>
<td>80</td>
<td>0.16</td>
<td>87</td>
</tr>
<tr>
<td>n = 15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cutter</td>
<td>0.67</td>
<td>100</td>
<td>0.91</td>
<td>100</td>
<td>0.89</td>
<td>100</td>
</tr>
<tr>
<td>n = 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lister: Cutter 11:1</td>
<td>2:1</td>
<td>5:1</td>
<td>1.25:1</td>
<td>5:1</td>
<td>1.1:1</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 10.3 Antibody response to 0.1ml HDCS vaccine given ID (Group 7) and 1.0ml given IM (Group 4) on days 0, 3, 7, and 14 (left hand panels), compared with the effect of 20 IU/kg Lister HRIG given on day 0 with ID vaccine therapy (Group 2) and IM vaccine therapy (Group 3) (right hand panels). Dots represent individual titres, and the solid line connects the GMTs.
Fig. 10.4 Antibody levels following passive administration of 20 IU/kg Lister HRIG only (Group 1) and with 1.0ml quantities of HDCSV given on days 0, 3, 7, and 14 (Group 3) are shown in the left hand panels. The titres following administration of Cutter HRIG only (Group 5), and with 1.0ml quantities of vaccine (Group 6) are shown on the right. The dots represent individual titres and the solid line connects the GMT.
12 Study 2 - Discussion

The results demonstrated that the kinetics and magnitude of the antibody response to 4 x 1.0 ml IM doses of HDCS vaccine were not statistically different from the response to one-tenth the amount administered intradermally. However, the size of the groups was small and it is acknowledged that this could readily mask real differences between the responses to the regimens under study. Indeed, on day 14 the GMTs of the vaccine only groups [group 7 (0.1ml ID) and group 4 (1.0 ml)] differed by more than two-fold (12.0 versus 28 IU/ml), as did the GMTs of the ID groups with and without HRIG (groups 7 and 2; 12 and 6.3 IU/ml respectively). Similarly the GMTs of groups 3 and 6 receiving 4 x 1.0 ml IM doses of HDCS and HRIG were lower than group 4 receiving 4 x 1.0 ml IM doses of vaccine only; moreover, one-way analysis of variance of the day 14 titres of the 5 vaccine groups under study revealed a statistically significant difference. Many factors correlate with host resistance to rabies infection. In dogs and cats that have been vaccinated against rabies, the titre of neutralizing antibody is related to protection after a rabies virus challenge: a neutralizing antibody titre of approximately 0.5 IU/ml at the time of challenge is necessary for uniform protection (Sikes et al, 1971b; Bunn et al, 1984). Similar data do not exist for man, and it is possibly misleading to emphasize statistically significant differences in antibody titre when all groups have high GMTs by day 14.

Other investigators have studied the effect of passive immunisation on the active antibody response to 1.0 ml quantities of HDSCV. Hafkin et al, (1978) and Kuwert et al, (1978b) showed that passive immunisation had no immunosuppressive effect; Mertz et al (1982) similarly obtained no evidence of immunosuppression, but the groups were not strictly comparable. Like ourselves Keller et al, (1977), Steck et al, (1978), and Hefti et al (1979) showed that the titres were consistently lower, but not significantly so, after passive immunisation. In a much larger study Méan et al (1978) compared day 30 antibody titres of 151 Swiss subjects undergoing post exposure treatment with and without antibody, and
observed significantly lower titres in the sero-vaccine group. They attributed this to vaccine of low potency (antigenic value 1.6), but in a subsequent study, Méan again found consistently lower titres in sero-vaccine groups, even when batches of adequate potency were used (Méan, 1980). Moreover, it appeared that there was indeed a relationship between vaccine potency and the suppressive effect of passive immunisation. Overall, it is evident that 20IU/kg of HRIG has only a modest immnosuppressive effect on the stimulus provided by 1.0 ml quantities of vaccine.

In the present report, detectable antibody was present in only 47-80% of subjects 24-48 hours after the administration of Lister HRIG but in 100% and at much higher titres 24 hours after receiving Cutter HRIG. Although 2.7 times the volume of Cutter RIG was given, which may influence absorption, it is difficult to understand why titres found in the Cutter HRIG groups were 5 to 11 times greater. The results suggest that either the Lister HRIG was of lower, or the Cutter HRIG of higher potency, than the quoted value.

The latter explanation is most likely, since the antibody levels observed after passive immunization with Lister HRIG were similar to those expected after the administration of 20 IU/kg. Between 45 and 52% of the body's IgG is found in the intravascular compartments (Waldman et al, 1971). Equilibration of passively administered IgG occurs rapidly, within 24 - 48 hours 50% escapes into the extravascular fluid compartments (Soothill, 1968; Waldmann & Strober, 1969). With a mean plasma volume of 41 ml/kg (Documenta Geigy, 1962), 50% of the total HRIG administered to a 66 Kg subject (the average weight in this study) would be distributed in 2.7 litres. Rabies immunoglobulin administered at 20IU/kg would have a theoretical concentration of 0.25 IU/ml after 48 hours, assuming complete absorption and normal distribution. The geometric mean of the observed values for subjects receiving Lister HRIG was 0.18 IU/ml, whereas in those receiving Cutter HRIG it was 0.91 IU/ml, almost 4 times its theoretical maximum value. Low or absent antibody titres, similar to those reported here, have been noted by other
workers (Keller et al, 1977; Kuwert et al, 1978b; Mertz et al, 1982; Klietman et al, 1980) in subjects passively immunised with 20IU/kg. Furthermore, retitration of Cutter HRIG by Dr George Turner showed the titre to be 2.5 times its quoted potency, which supports these clinical observations.

If detectable antibody within the first 48 hours is a criterion of protection, then 20 IU/kg HRIG may be inadequate and the larger dose more appropriate, particularly in the presence of a potent vaccine such as HDCSV. In Hafkins study (1978), HRIG was was followed by four doses of HDCSV administered SC. They initially administered what was thought to be 20 IU of HRIG/kg; 6 and 8 of 25 volunteers had, respectively, no detectable antibody or only minimal levels two days later. Subsequently, the HRIG was retitrated and found to have a titre of about 12 IU/kg. Subsequent studies were done with HRIG of higher titre. All the volunteers given 20 or 30 IU of HRIG/kg developed adequate active antibody responses to HDCSV.

In view of the immunogenicity of HDCSV and the inaccuracies of the present methods of estimating the potency of HRIG (Fitzgerald et al, 1975), which, even after replicate titrations, are only just capable of distinguishing between 20 and 40 IU/ml, the data presented here and the results of others suggest that trials of larger quantities e.g., 30 or 40 IU/Kg, of HRIG in combination with HDCSV are warranted.

13 Studies 3 to 6 - Interferon response to human diploid cell strain rabies vaccine in man

Early postexposure trials with HDCSV in Iran (Bahmanyar et al, 1976) and Germany (Kuwart et al, 1977) showed that HDCSV protects man after bites by proven rabid animals. Studies in experimental animals show that rabies can often be prevented if interferon is administered or induced before or after the time of infection (Baer et al, 1977; Baer & Yager, 1977; Fenje & Postic, 1970; Fenje & Postic, 1971; Harmon & Janis, 1975; HIlfenhaus et al, 1975; Janis & Habel, 1972; Kaplan et al, 1962; Postic & Fenje, 1971; Turner, 1972; Vieuchange,
Inactivated Kern Canyon virus, influenza B virus and some tissue culture rabies vaccines are capable of inducing interferon and protecting animals against challenge with rabies virus, but in these studies the rabies vaccines gave no protection if interferon could not be detected (Baer & Yager, 1977; Wiktor et al, 1972a). As interferon may have an important role in the postexposure treatment of man, studies 3 - 6 were undertaken to determine whether it is regularly induced by HDCSV.

14. Titration of interferon

Interferon titres expressed in units per ml were calculated by comparison with the MRC Research Standard B 69/19 human leucocyte interferon which was titrated in each assay. The mean reduction of the VSV plaque count by 1 unit of interferon was 45.5% (range 30.1 - 84.7; standard deviation 12.2) and there was a high degree of reproducibility (Figure 10.5). Only serum dilutions which reduced the mean plaque count beyond the 95% confidence limits of the VSV control plaque counts in 6 to 18 wells were considered to have antiviral activity. This generally limited the sensitivity of the assay to 0.5 to 0.9 units of interferon per ml of serum dilution. Samples were screened at a 1 in 10 dilution.
Fig. 10.5 Percentage reduction in VSV plaque counts following pretreatment of GL-V3 cells with 1 unit of MRC Research Standard B, 69/19, human leucocyte interferon.
15. Study 3

Ten volunteers who received 0.1 ml of HDCSV (Lot M 1200; antigenic value 4.1) intradermally and 14 who were given 1.0 ml IM were bled for assay of interferon activity immediately before and 24 and 48 hours after immunisation; all were participants in Study 2 and none had previously been immunised against rabies.

None of 10 who received 0.1 ml ID developed an interferon-like response. However, low titres of antiviral activity were consistently found in sera taken from 4 people 24 hr after 1.0 ml was given IM (Table 10.21); such modest titres prevented its characterisation as interferon. Nonetheless, absence of antiviral activity from samples taken before vaccination and the failure to show any antiviral effect on mouse L929 cells, in contrast to mouse standard interferon, both suggested that an interferon response had occurred.

Table 10.21 Study 3; Interferon-like activity found after vaccination with 0.1 ml ID or 1.0 ml IM.

<table>
<thead>
<tr>
<th>Dose and route of vaccination</th>
<th>No. (+)ve/total</th>
<th>Equivalent titre of interferon (U/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 ml ID</td>
<td>0/5</td>
<td>-</td>
</tr>
<tr>
<td>0.1 ml ID + 20 IU/Kg HRIG</td>
<td>0/5</td>
<td>-</td>
</tr>
<tr>
<td>1.0 ml IM + 20 IU/Kg HRIG</td>
<td>2/9</td>
<td>7, 8</td>
</tr>
<tr>
<td>1.0 ml IM</td>
<td>2/5</td>
<td>7, 7</td>
</tr>
</tbody>
</table>

Note: * Each value represents the mean of 4 titrations

16. Study 4

The ability of HDCSV to induce interferon was studied further using a different batch of vaccine with a higher antigenic value (Batch No. M 1015; A.V. 6.9). In this study blood samples were collected before and 24 hours after IM injections with 1.0 ml of
vaccine on days 0 and 28 administered to eight animal technicians for pre-exposure prophylaxis; one subject had been vaccinated previously against rabies. None of the sera taken before vaccination reduced the VSV plaque count significantly. By contrast low levels of interferon-like activity (8-13 U/ml) were found in the 21 hour sera of 6 of 7 subjects with no previous vaccination against rabies. No antiviral activity was found in the sera of one person who had been vaccinated before or in any of the 8 subjects after the day 28 injection. Repeat testing on mouse L929 cells failed to show any antiviral activity, suggesting that the activity was due to human interferon. These results suggest that interferon is induced by primary vaccination with HDCSV, but not by revaccination, and are in agreement with those found in rabbits (Wiktor et al, 1972c).

17. Study 5
Prompted by the encouraging results of an earlier study in which four ID injections were given on a single occasion (Turner et al, 1976), and with the ultimate goal of abbreviating rabies postexposure treatment to one or two injections only, I gave 1.0ml of HDCSV (Batch No. N 0337; A.V. 3.5) to two volunteers at eight sites on the medial and lateral aspects of the upper arms and thighs and collected blood samples for tests of interferon activity before and 6, 12, 18, and 24 hours after vaccination. Titres of virus neutralising antibody were measured on blood samples collected on days 0, 3, 7, 10, 14, 21, 30, 50, and 90. Neither volunteer had previously been vaccinated against rabies.

Assay for antiviral activity on GL-V3 cells showed that both vaccinees developed low titres of interferon-like activity in samples collected 12-24 hours after vaccination (Table 10.22). Although the titres of interferon-like activity were similar to those found in the previous studies, the antibody titres attained after multisite-ID vaccination were some 6-fold higher on day 30 than after IM vaccination with the same volume of vaccine of higher antigenic value (Batch No. M 1015; A.V. 6.9) given as a single IM injection (Figure 10.6). Review of the
Table 10.22. Study 5; Titres of circulating antiviral activity in two subjects given 1.0ml of HDCSV at 8 separate ID sites.

<table>
<thead>
<tr>
<th>Vaccinee</th>
<th>Titre of interferon (u/ml) at time (hrs) after vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
</tr>
</tbody>
</table>

* Not significant; plaque count reduced, but not beyond the 95% confidence limit for controls

Fig. 10.6 GMT of rabies virus neutralizing antibody (IU/ml) of volunteers immunized with HDCSV. Left, comparison of titres in groups given vaccine alone and vaccine plus HRIG on day 0; right, comparison of titres in groups given 1.0ml vaccine ID in 8 sites and IM in a single site.
antibody response in these two volunteers showed that the titres were much higher than those following 4 IM doses on days 0, 3, 7, and 14; by day 7, the titres were approximately 15-fold higher with the multisite ID regimen and a peak GMT of 355 IU/ml was found on day 14. Thus the results of studies 3, 4 and 5 indicate that there is poor correlation between the neutralising antibody response and induction of interferon-like activity. Study 5 further showed that substantial titres of antibody remain in the circulation for periods of 90 days or more after multisite ID vaccination.

Study 6

The low titres of antiviral activity found in the previous studies prevented characterization of the active compound as interferon. This became possible, however, when sera from subjects in Germany became available for analysis. The sera were from 53 volunteers who had been recruited by Professor Kuwert in Essen to a dose-response study using Mérieux and Behringwerke HDCSV.

HDCSV Lot NO691 (Mérieux) was prepared from the supernatants of WI-38 cells infected with the Pitman-Moore (PM) strain of fixed rabies virus, concentrated by ultrafiltration and inactivated with β-propiolactone (titre of infectious virus before inactivation = \(10^{6.5}\) ICLD\(_{50}\)/ml, concentration factor = 20, final protein content 68mg/1.0ml vial; date of production, July 1976). HDCSV Lot 760205-2 (Behring) was prepared by continuous flow ultracentrifugation of supernatants of MRC-5 cells infected with the PM strain and inactivated with β-propiolactone (titre of infectious virus before inactivation = \(10^{7.5}\) ICLD\(_{50}\)/ml, concentration factor = 8, final protein content 7.4mg/1.0ml vial; date of production, February 1976). The 53 volunteers were divided into 10 study groups (A - J). The groups were vaccinated intramuscularly (Gluteus maximus muscle) with either the Mérieux or Behring vaccine according to the schedules shown in Table 10.23. Volunteers who had not been previously vaccinated (Groups A - H) were either given...
Table 10.23  Study 6; Schedules of immunization and number of vaccinees in each study group.

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>Vaccine</th>
<th>Antigenic value</th>
<th>No. of volunteers</th>
<th>Previous rabies vaccination</th>
<th>Volume of 1st vaccine dose (ml)</th>
<th>Total No. vaccine doses</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Mérieux</td>
<td>5</td>
<td>5</td>
<td>No</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>B</td>
<td>Mérieux</td>
<td>5</td>
<td>6</td>
<td>No</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>C</td>
<td>Mérieux</td>
<td>5</td>
<td>5</td>
<td>No</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>D</td>
<td>Mérieux</td>
<td>5</td>
<td>6</td>
<td>No</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>E</td>
<td>Behring</td>
<td>13</td>
<td>5</td>
<td>No</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>F</td>
<td>Behring</td>
<td>13</td>
<td>5</td>
<td>No</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>G</td>
<td>Behring</td>
<td>13</td>
<td>5</td>
<td>No</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>H</td>
<td>Behring</td>
<td>13</td>
<td>5</td>
<td>Yes</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>I</td>
<td>Behring</td>
<td>13</td>
<td>5</td>
<td>Yes</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>J</td>
<td>Mérieux</td>
<td>5</td>
<td>6</td>
<td>Yes</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

Antigenic value determined by the NIH potency test (geometric mean of 13 assays for Mérieux vaccine, Lot N0691, and 4 assays for Behring vaccine, Lot 760205-2).

5 or 6 doses of vaccine. Those receiving 6 doses were vaccinated on days 0, 3, 7, 14, 28 and 84; in the 5 dose schedule the vaccination on day 3 was omitted. In these groups the first dose was either 1.0ml or 3.0ml and all subsequent doses were of 1.0ml. The previously vaccinated groups (I and J) were given a single booster dose of 3.0ml. Serum samples were obtained immediately before, and 6, 17, 25, 37, 64, and 88 hours after, the first vaccine dose and were coded and stored at -20°C until assayed for interferon.

Peak titres of interferon developed 17-25 hours after primary immunization (Figure 10.7). Although a pronounced dose-response effect was observed, the two vaccines differed in their ability to induce interferon; interferon was detected in all 11 subjects given 3.0ml of Mérieux vaccine (Groups A and B, mean titre 97 units/ml), and in 9 of 11 vaccinees after 1.0ml vaccine (Groups C and D, mean titre 28 units/ml). By comparison, the Behring vaccine was a poor
Fig. 10.7 Time course of individual and mean interferon responses to HDCS rabies vaccine in unprimed vaccinees.
interferon-inducer, significant titres were found in 9 of 10 vaccinees given 3.0ml (Groups E and F, mean titre, 26 units/ml) and in only 1 of 10 given 1.0ml (groups G and H, mean titre 1.6 units/ml). The interferon response was diminished after the second (1.0ml) dose of either vaccine. Seven of 12 persons given a 1.0ml booster with Mérieux vaccine (Groups B and D) developed rising titres of antiviral activity but neither the titres that were attained or the proportion of persons with antiviral activity were significantly greater than in those groups not given a second injection (Groups A and C; Chi-square test and unpaired t-test). After a second injection of Behring vaccine, only one of 10 vaccinees developed an interferon-like response. Interferon was generally absent after 3.0ml booster doses, and was found in only 2 of 5 vaccinees given boosters with Behring vaccine (10 and 18 units/ml), while no volunteers (0/6) given Mérieux vaccine developed significant titres (Groups I and J).

To identify the antiviral activity as interferon, I prepared a serum pool containing high-titred samples. The inhibitor was shown to be interferon on the basis of its sensitivity to trypsin, stability at pH 2 and its non-sedimentable and heat-labile properties.

19. Studies 3 - 6, Discussion

Studies 3 - 6 showed that human inoculation with HDCSV is often followed by by the appearance of a non-specific viral inhibitor in the serum. This substance is inactive on non-primate cells and has been identified as interferon on the basis of its sensitivity to trypsin, stability at pH 2 and its non-sedimentable and heat-labile properties. Interferon occasionally appeared in the serum as early as 6 hours after vaccination but the response was maximal at 17 to 25 hours. Induction of interferon was related to the dose of vaccine given, but was independent of its antigenic value and the titre of infectious virus before inactivation. Schelermann et al (1987) recently described similar dose-related interferon kinetics using 1.0 and 3.0ml
doses of purified chick embryo cell-culture vaccine. At the 1.0ml dose level, only 2 of 11 PCECV recipients developed an interferon response 20 hours after vaccination as compared with 7 out of 10 who received 3.0ml. Bijck et al (1984) found no interferon in 31 subjects after administration of 1.0ml PCECV.

The ability of the HDCS vaccines to induce interferon was not lost after prolonged storage and the less purified, Mérieux vaccine prepared from infected WI-38 cells induced the higher titres. In contrast to primary vaccination, interferon was generally not induced by boosters, a finding which is in agreement with observations in laboratory animals (Wiktor et al, 1972b; Mifune et al, 1980; Pille & Matevoysan, 1985) and which emphasizes the independence of the humoral and interferon responses. Fornosi et al (1985) similarly found that the interferon titres evoked by Mérieux HDCS vaccine on day 14 were on average 80% lower than those induced on day 3. However, high titres of interferon were again found after the day 90 vaccination when circulating virus neutralizing antibody titres were high.

The importance of an inconsistent vaccine-induced interferon response in postexposure protection is questionable. Although most animal studies show that interferon gives incomplete protection against rabies, levels in the serum of 20U/ml have been protective (Wiktor et al, 1972b) and it is probable that even the modest levels of activity found in these studies are of value. However, a postexposure protection study in non-human primates at the US Center for Disease Control suggests that interferon production by HDCSV is not a marker for survival. In this study, HDCSV induced low titres of interferon in 5 of 8 rhesus monkeys and the outcome of infection, i.e., death or survival, was in this small number of animals unrelated to the interferon response (Baer et al, 1979). Similarly, Pille & Matevoysan (1985) studied two rabies vaccines, only one of which induced interferon, and found no association between interferon induction and post-exposure protection in mice. The importance of vaccine induced interferon is further questioned by the general observation that post-exposure administration of HDCSV or PCECV is almost always protective, yet the interferon response to the new tissue
culture vaccines is so variable. Nonetheless, Baer et al (1979) found that a combination of vaccine and interferon inducer was as effective as hyperimmune serum and vaccine, and much better than vaccine alone. It may be concluded that interferon is a valuable *adjunct* to rabies postexposure therapy, but the ability of vaccines to induce interferon does not appear to be essential for protection.

20. *In-vitro* transformation of lymphocytes from vaccinees

Both humoral and cell-mediated immunity are important in protecting animals from viral infections and in ridding the infected host of viruses (Blanden, 1974; Bloom & Rager-Zisman, 1975; Woodruff & Woodruff, 1975). Viral antibody in serum has usually been used to measure the immune status of the host against viruses, generally because of the ease of viral antibody assays and the correlation between the presence of antibody and resistance to viruses that have a viraemic phase. Cell-mediated responses may be more appropriate guides to the hosts' immune status, especially when the virus is mainly cell-associated, as in rabies.

Three types of *in vitro* test have generally been employed to study cell-mediated responses to viruses, namely lymphocyte stimulation (blast transformation) by sensitized cells in response to viral antigens of virus infected cells; production of lymphokines (macrophage inhibitory factor, leukocyte inhibitory factor) by sensitized lymphocytes in response to viral antigens; and cell-mediated cytotoxicity of virus-infected target cells by immune lymphoid cells. Studies 7 and 8 were undertaken to establish whether lymphocyte transformation regularly occurs after immunization with HDCSV.

21. Study 7, *In-vitro* transformation of lymphocytes following vaccination

Blood samples for tests of lymphocyte transformation and determination of levels of virus neutralizing antibody, measured by the interference inhibition test, were
taken on day 30 after primary vaccination from 10 of 70 veterinary students who took part in study 2. Five others were given 1.0ml HDCSV intramuscularly on days 0, 3, 7 and 14; five other volunteers received HRIG in addition to the first dose of vaccine on day 0 and vaccine alone on days 3, 7, and 14. No volunteer had previously received antirabies therapy.

Among the blood samples obtained on day 30, eight demonstrated lymphocyte stimulation by the HDCS antigen (Table 10.24); four of these were in the vaccine only group and four in the group given vaccine and HRIG. Thus lymphocyte transformation was not suppressed by passive immunization with 20 IU of HRIG per Kg administered with the first vaccine dose. No correlation was found between the lymphocyte transformation increment and the antibody titre, but the stimulation increments tended to be greater in the group given vaccine and HRIG.
Table 10.24  Study 7; Increments in lymphocyte transformation and titres of rabies virus-neutralizing antibody on day 30 for 10 volunteers vaccinated with a human diploid cell strain rabies virus vaccine, as compared with responses of unvaccinated controls.

<table>
<thead>
<tr>
<th>Vaccination procedure</th>
<th>Increment (cpm) in lymphocyte transformation over control cultures in response to Neutralising antibody titre (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHA</td>
</tr>
<tr>
<td></td>
<td>(PHA)</td>
</tr>
<tr>
<td>1.0ml IM on days 0,3,7, and 14</td>
<td>52 667</td>
</tr>
<tr>
<td></td>
<td>105 842</td>
</tr>
<tr>
<td></td>
<td>61 362</td>
</tr>
<tr>
<td></td>
<td>59 936</td>
</tr>
<tr>
<td></td>
<td>82 929</td>
</tr>
<tr>
<td>1.0ml IM on days 0,3,7, and 14 + 20 IU of HRIG/Kg on day 0</td>
<td>48 766</td>
</tr>
<tr>
<td></td>
<td>92 672</td>
</tr>
<tr>
<td></td>
<td>47 444</td>
</tr>
<tr>
<td></td>
<td>56 740</td>
</tr>
<tr>
<td></td>
<td>84 001</td>
</tr>
<tr>
<td>None (cord blood lymphocyte controls)</td>
<td>72 437</td>
</tr>
<tr>
<td></td>
<td>101 312</td>
</tr>
<tr>
<td></td>
<td>91 711</td>
</tr>
<tr>
<td></td>
<td>57 231</td>
</tr>
<tr>
<td></td>
<td>86 399</td>
</tr>
<tr>
<td></td>
<td>67 851</td>
</tr>
<tr>
<td></td>
<td>98 235</td>
</tr>
<tr>
<td></td>
<td>49 121</td>
</tr>
<tr>
<td></td>
<td>90 005</td>
</tr>
<tr>
<td></td>
<td>77 413</td>
</tr>
</tbody>
</table>

NOTE. Between the lymphocyte transformation increment and antibody titre, $r = 0.22$. PHA = phytohaemagglutinin, IU = international units, and HRIG = human rabies immune globulin. - = not assayed. *Significant increment in lymphocyte transformation (>3 000 cpm).
22. Study 8, Antibody production and *in-vitro* transformation of enriched T-lymphocytes following multisite ID vaccination

Because enriched T-cell populations were not used in study 7, it was possible that the bone marrow derived B-lymphocytes were the only ones responding to the rabies antigen. In study 8, the T and B lymphocyte sub-populations were enriched so as to determine whether blast-transformation is a T-cell response. HDCS vaccine (0.8ml) (L'Institut Mérieux; Lot R0220; antigenic value 10.8) was given intradermally on a single occasion to 10 volunteers at 8 sites on the medial and lateral aspects of the upper arms and thighs. Blood samples for tests of lymphocyte transformation, rabies neutralizing antibody, and rabies IgG antibody were taken before vaccination and 10, 14, 21, and 42 days later. Further blood samples for antibody titration were taken on days 0 and 100. Four subjects were vaccinated with a Dermojet injector (Krantz, 1959); the six remaining volunteers were given vaccine with a 25-guage needle and tuberculin syringe. No volunteer had received antirabies vaccine previously.

Because of the pH indicator in the vaccine, needle inoculation resulted in the immediate formation of magenta-coloured skin blebs at each injection site. Vaccination with the Dermojet injector was generally quicker, but bleb formation was less satisfactory and in areas where the skin is especially soft, i.e., the medial aspects of the thighs and arms, it appeared that most, if not all, of the vaccine had entered the subcutaneous tissues. This method of inoculation was also associated with substantially lower titres of neutralizing and IgG antibodies than after needle inoculation (Figures 10.8 and 10.9), particularly on days 10 and 14, but because of the small number of subjects in each group, the difference was not significantly different. However, comparison of the neutralizing antibody titres for days 10, 14, and 21 combined showed a significant difference between the GMTs of 2.04 for the Dermojet injector group, and 7.07 for the group given vaccine by needle and syringe. Substantial antibody titres developed in the 6 subjects who were
Fig. 10.8 Individual and geometric mean titres of rabies neutralizing antibody after inoculation with needle and syringe (open circles, solid line) and dermojet injector (closed circles and broken line).
Fig. 10.9 Individual and geometric mean titres of rabies IgG antibody (established by ELISA) after inoculation with needle and syringe (open circles, solid line) and dermojet injector (closed circles and broken line).
inoculated with a needle and syringe. By 10 days, neutralizing antibody titres ranged between 1.3 and 8.7 IU/ml (GMT, 3.0 IU/ml) and antirabies IgG ranged between 1/345 and 1/1250 (GMT 1/693). Peak titres of virus neutralising antibody were found on day 14 (GMT 14.3 IU/ml), but substantial titres were still present 100 days after vaccination.

The increments (cpm) in lymphocyte transformation are expressed as a ratio to the cpm values of non-stimulated control cultures (Table 10.25). The results show that T-lymphocytes from 2 of 9 vaccinees were significantly stimulated *in vitro* on day 14, and there were similar significant increments on days 21 and 42 with T-lymphocytes from 6 of 8, and 8 of 10 subjects respectively. This blast transformation occurred with cells from 3 of 4 people given vaccine by the Dermojet injector, and from 5 of 6 people inoculated with a needle and syringe. There was no significant difference between the transformation increments in either group, and no significant correlation was found between the transformation increment and the titre of antirabies IgG or neutralising antibody, even when the data from the previous study were included. None of the enriched B-cell cultures underwent blast transformation in response to the rabies antigen.
Table 10.25  Study 8; Mitogen and antigen stimulation of peripheral blood lymphocytes obtained 0, 10, 14, 21, and 42 days after vaccination of ten volunteers with 0.8 ml of HDCS rabies-virus vaccine.

<table>
<thead>
<tr>
<th>Inoculated by</th>
<th>Lymphocyte stimulation index*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 day</td>
</tr>
<tr>
<td></td>
<td>PHA</td>
</tr>
<tr>
<td></td>
<td>T</td>
</tr>
<tr>
<td>Inoculated by</td>
<td></td>
</tr>
<tr>
<td>needle and</td>
<td></td>
</tr>
<tr>
<td>syringe</td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>79</td>
</tr>
<tr>
<td>b</td>
<td>91</td>
</tr>
<tr>
<td>c</td>
<td>47</td>
</tr>
<tr>
<td>d</td>
<td>58</td>
</tr>
<tr>
<td>e</td>
<td>63</td>
</tr>
<tr>
<td>f</td>
<td>102</td>
</tr>
<tr>
<td>Inoculated by</td>
<td></td>
</tr>
<tr>
<td>dermo-jet</td>
<td></td>
</tr>
<tr>
<td>injection</td>
<td></td>
</tr>
<tr>
<td>g</td>
<td>54</td>
</tr>
<tr>
<td>h</td>
<td>83</td>
</tr>
<tr>
<td>i</td>
<td>95</td>
</tr>
<tr>
<td>j</td>
<td>-</td>
</tr>
<tr>
<td>Unvaccinated</td>
<td></td>
</tr>
<tr>
<td>k</td>
<td>86</td>
</tr>
<tr>
<td>Cord blood</td>
<td></td>
</tr>
<tr>
<td>l</td>
<td>105</td>
</tr>
</tbody>
</table>
23. Studies 7 and 8, discussion

Lymphocyte transformation occurred with peripheral lymphocytes taken from 8 of 10 volunteers (Study 7) after the first four 1.0ml doses of the current, WHO-recommended, post-exposure regimen for HDSV. Passive immunization with 20 IU of HRIG per kg on day 0 did not suppress the antigenic stimulation of lymphocytes. Although it could not be concluded that the transformation occurred primarily with T-lymphocytes, there was no correlation between the antibody titre and the transformation increment. By contrast, transformation of spleen cells, not thymus cells, occurs in rabbits after immunization with live or inactivated rabies virus, and a correlation exists between the transformation increment and in vitro production of antibody (Wiktor, et al, 1974).

Separation of the lymphocyte sub-populations in study 8 revealed that blast transformation after HDSV is a T cell response. Furthermore it occurred in the same proportion of vaccinees as was found in the previous study, but with only one quarter the volume of vaccine. Clearly, if high titres of neutralising antibody and a cell-mediated response to vaccination are both important for protection, the present study indicates that they can be obtained equally well and with much smaller quantities of vaccine than are used at present.

More recent studies confirm and extend the above findings. Bijok et al (1985) reported blast transformation indices of >2 using cells from 5 of 7 vaccinees 30 days after 3.0ml of PCECV. Ratanavongsiri et al (1985) similarly evaluated the lymphocyte transformation responses following intradermal vaccination with either 0.1ml x 4 on days 0, 3, and 7 and 0.1ml on days 28 and 91, or 1ml HDSV by the IM route on days 0, 3, 7, 14, 28 and 91. By day 7, 7 of 10 in the ID group had stimulation indices >2 as compared with 0 of 6 in the IM group. Maximum stimulation occurred 14 days after the first ID injection and was delayed until day 28 with IM vaccination. No stimulation was detected using specimens collected on the 100th day post-vaccination. The authors confirmed that blast
transformation occurred earlier in response to multisite ID vaccination as compared with the conventional IM regimen in subsequent studies with vero cell culture vaccine (Phanuphak et al, 1987).

Blast transformation responses are not unique to recipients of potent tissue culture vaccines. Veiga et al (1987) showed that lymphocytes from 3 of 3 vaccinees underwent blastogenesis when collected 10 days after primary vaccination with SMBV; the transformation indices peaked on day 14, but significant transformation was still evident on day 35. Eleven people were studied following boosters with SMBV. Blastogenesis occurred with lymphocytes from all 11 subjects when bled on days 3 to 7 and 35, and persisted, in some cases, until day 110. Santos et al (1980) used migration inhibition tests and skin testing to study cellular immunity in 6 unvaccinated controls and three people given 10 daily doses of SMB. Positive skin tests were observed on day 5, the maximum reactivity occurred on days 16-20, and positive results were still obtained on day 28. Leukocyte migration inhibition developed somewhat later on day 15, remained positive until day 30, and declined to non-significant levels by day 36. Migration inhibition has also been documented in rabbits given a live avian rabies virus vaccine (Ramanna & Pal, 1980). Maximal inhibition was seen on the 6th day post-vaccination, the effect was still evident on day 14, but was no longer detectable 17 days after vaccination. The proportion of animals responding was not stated.

Studies 7 and 8 (Nicholson et al, 1979, 1981) and subsequent reports in the literature demonstrate that cellular responses develop in most, possibly all, vaccinees given potent rabies vaccines. Although the height of the cellular response and its persistence are not correlated with antibody levels, it appears that antibody production is generally accompanied by a measurable cellular response. The contribution of cellular immunity to protection afforded by human post-exposure immunization remains inconclusive, but it can reasonably be speculated that it is of considerable benefit and may even be essential.
Study 8 confirmed the observation in Study 5 that 8 x 0.1ml injections given in separate ID sites on one occasion induce high titres of virus neutralizing antibody rapidly. It was further shown that the early production of virus neutralizing antibody is accompanied by high titres of antirabies IgG. This early IgG response may be most important as it is now well established that neutralizing antibody of the IgG class, unlike IgM neutralizing antibody, confers protection upon experimental animals challenged with rabies (Turner, 1978, Mifune et al, 1980), and may be of prime importance in the post-exposure treatment of man.

Opponents to the administration of rabies vaccine by the ID route claim that it is technically difficult, especially in the elderly and the very young. Vaccination with a jet injector can be achieved more rapidly than with a needle and syringe, it requires less skill, and has been used successfully over the years with various vaccines (Parker, 1948; Research Committee of the British Thoracic and Tuberculosis Association, 1971; Stanfield et al, 1972; Payler & Skirrow, 1974). Accordingly the two methods of vaccination were compared in a pilot study. Bleb formation was poor using the injector, suggesting that much of the vaccine went subcutaneously, and the titres of IgG and neutralizing antibody were both lower. Shortly after publication of these findings (Nicholson et al, 1981), Bernard et al (1982b) reported a comparison of the antibody responses to 0.1ml doses of HDCSV administered on days 0, 7, and 28, either by the ID route using a needle and syringe or jet injector, or by the subcutaneous route. After ID inoculation by needle and syringe the titres on day 49 were superior to those following both jet injection and subcutaneous administration (GMT's 7.4 versus 3.05 and 3.17 respectively). In both studies the titres after jet injection were approximately two-fold lower than with a needle and syringe and Bernard et al (1982b) also concluded that jet injection delivered most of the vaccine subcutaneously. Nevertheless, these authors considered that the injector deserved further study, but in view of the risks of transmission of hepatitis B virus, human immunodeficiency virus, and other pathogens, use of the jet injector is now considered wholly inappropriate.
24. Study 9, Study of the responses to ID vaccination at British Airways Immunization Centre

Since 1980, the British Airways immunization Centre has offered travellers to the tropics pre-exposure anti-rabies vaccination using two ID doses of HDCSV, separated by an interval of 4 weeks, and a booster at 12 months. In 1980, the intradermal route was not universally accepted and the British Airways venture provided the opportunity of assessing the efficacy of ID vaccination at a busy immunization centre. Seventy coded sera were provided by Dr J Furlong at British Airways for antibody titration by ELISA. Endpoint titres were calculated as described in the Methods chapter and in this early study with ELISA a titre of ≥1:10 was taken as positive for rabies antibody.

No rabies IgG antibody was found in 12 sera taken from non-vaccinated controls. By contrast, 40 of 43 (93%) samples taken three to six weeks after primary ID vaccination with 0.1ml of HDCS vaccine contained antibody ranging from 1:19 to ≥ 1:12 500 (GMT ≥ 1:520). Similarly the eight samples taken from subjects 4-12 weeks after revaccination (0.1ml ID) contained antibody ranging from 1:1025 to ≥ 1:12 500 (GMT ≥ 1:4367). Five persons who had received HDCS vaccine one to two years previously had titres ranging between 1:25 to 1:2,855 (GMT 1:186). By contrast, two persons vaccinated recently in the Soviet Union and Bolivia had no detectable rabies antibody.

25. Study 9, Discussion

The 93% seroconversion rate obtained in a busy immunization centre with many different batches of vaccine was virtually identical to the 98% response in Study 1. This observation together with the high titres found after revaccination clearly demonstrate that the ID route can be used successfully within busy immunization centres.
26. Study 10, Postexposure multisite ID administration of HDCSV in rabbits

Although studies 5 and 8 and a previous study using 4 x ID sites (Turner et al., 1976) had together established the antigenicity of multisite ID vaccination, it was felt that a human post-exposure trial of multisite ID vaccination would be more acceptable if the regimen was of proven efficacy in laboratory animals. Because rabbits are comparatively inexpensive and readily available (in contrast to non-human primates), I titrated an arctic fox rabies virus isolate in rabbits to establish its LD$_{50}$ for this species.

The nuchal muscles of 56 New Zealand White rabbits were then inoculated with 50 rabbit LD$_{50}$ of a first mouse brain passage of the arctic fox rabies virus isolate in two separate sites (0.5ml each side). Eight hours later, 14 rabbits were given 1.0ml of HDCSV IM into the left forelimb, 14 received 4 x 0.2ml of vaccine ID into each limb, 14 received 1.0ml of vaccine intravenously, and the remaining 14 were used as controls and received no prophylaxis. Each animal was observed over a 12 month period for signs of rabies. None of the rabbits had been exposed to rabies previously or had been immunised against the disease.

Nine of the 14 unvaccinated rabbits developed paralysis and died after infection with 50 rabbit LD$_{50}$ of street rabies virus. In these animals, forelimb paralysis developed within 13 to 38 days of infection and progressed to complete paralysis and death 2 to 7 days later. Post-exposure treatment with a single dose of HDCSV reduced the mortality significantly; the administration of 1.0ml of vaccine IM gave significant ($p = 0.018$, Fisher's exact test) but incomplete protection with 2 of 14 animals developing paralysis and dying after incubation periods of 13 and 20 days. None of the 14 rabbits died after receiving 4 x ID injections of 0.2ml of HDCSV into each limb ($p = 0.0006$, Fisher's exact test), and none died after intravenous administration of vaccine.
27. Study 10, Discussion
This study clearly demonstrates that 0.8 ml of vaccine, administered in 4 separate ID sites after infection with street rabies virus, affords significant protection against rabies. Similarly, 0 of 14 rabbits died after intravenous vaccination and 2 of 14 (14%) died after IM administration. The protection afforded by IM vaccination was similar to that in monkeys given a highly potent (antigenic value 49) Pitman Moore strain rabies vaccine prepared in BHK cells (Sikes et al, 1971a) and better than that in monkeys given HDCSV (Wiktor, 1971; Baer et al, 1979).

The results of the present study, together with the data on the antibody production, interferon induction, and blast transformation responses to multisite ID vaccination, was considered sufficient to justify a post-exposure trial of HDCSV by the ID route and accordingly approaches were made to several potential collaborators.

28. Study 11 - Stability of HDCSV rabies vaccine at high ambient temperatures

For HDCSV to be widely available to those most in need it must be able to withstand lengthy exposure to the high ambient temperatures that it might encounter during transit and storage in developing countries. This critical quality of HDCSV is the subject of Study 11, which was carried out under the auspices of WHO.

The following observations concern the laboratory potency and antigenicity in man of batches of HDCSV that were distributed and stored at different temperatures in Pakistan. The potency of the vaccine was measured originally in France, and subsequently in England by Dr GS Turner and Mr P Harrison by the NIH method; by single radial diffusion (Ferguson & Schild, 1982) at the National Institute of Biological Standards and Control by Dr M Ferguson; and by myself by an antigen-extinction test in mice which determines the dilution of vaccine giving an antibody titre of 1/100 as measured by ELISA and calculated by regression analysis. The vaccine underwent each test in London before the study began; repeat titrations were later made on test vaccine returned from Pakistan and on control vaccine stored.
throughout the duration of the project in London at 4°C. To assess its antigenicity for man, volunteers at three centres (Islamabad, Peshawar, and Hyderabad) were randomly allocated to groups receiving two doses of vaccine 28 days apart given either IM (1.0ml) or ID (0.1ml), and their rabies IgG levels on days 0, 14, and 42 were titrated by ELISA (day 0 and day 14 sera were titrated by the author in Pakistan, and day 42 sera were titrated in London, but 14 of the day 0 and day 14 samples were retitrated in London to assess comparability between the 2 centres).

The test vaccine (Lot V 0256) was hand-carried by air to Islamabad in early September in an insulated cool box. Shortly after arrival it was placed in cold-storage at the Rabies Vaccine Unit, National Institute of Health, and divided into three batches. Batch A was dispatched 4 weeks later by bus 110 miles to the Military Hospital in Peshawar, North West Frontier Province; it was packed in ice in an insulated container and arrived at its destination 6 hours later, where it was stored in an electrically-powered domestic refrigerator until required. In contrast, no special care was taken with batch B, which was packed in an ordinary cardboard container and then sent more than 1 000 miles by air and land via Karachi to the Military Hospital in Hyderabad, Sind Province. It arrived 11 days later and was stored in the dispensary at ambient room temperatures until required. Batch C, for use in Islamabad, was kept in cold-storage at the National Institute of Health until required. The first inoculations at each centre were given approximately 7 weeks after arrival of the vaccine in Pakistan. Maximum and minimum temperature recordings were usually made twice a day at each centre throughout the study.

Thirty volunteers from each centre gave informed consent for participation in the study. The vaccinees in Peshawar and Hyderabad were mostly members of the Army Medical Corps, whereas those in Islamabad were either students or staff at the National Institute of Health. Altogether, there were 86 males and 4 females, who were aged 13 to 55 years (mean 28, standard deviation 8.6 years). Approval for the study was given by the Harrow District ethical committee at Northwick Park.
29. Study 11 - Data

Temperature recordings - Batch A was dispatched to Peshawar from the National Institute of Health, Islamabad, after 29 days' storage at 4 to 8°C (mean 6.5°C; SD 0.6°C); the first inoculations were given after 22 days' storage in Peshawar at 2 to 13°C (mean 5.1°C; SD 3.0°C). Maximum and minimum temperature recordings are not available for the 11 days it took batch B to reach Hyderabad, but the maximum daily temperatures given in the Karachi newspapers during this period ranged from 35 to 40°C. The first injections with batch B were given after a further 40 days' storage at 26 to 36°C (mean 31.8°C; SD 1.9°C); the second dose was given after a total of 68 days' storage in Hyderabad at 24 to 36°C (mean 28.4°C; SD 3.2°C). The first injections with batch C were given in Islamabad after 49 days at 3 to 8°C (mean 6.5°C; SD 0.7°C) and the second after 77 days at 3 to 12°C (mean 6.3°C; SD 1.1°C).

Clinical reaction - The vaccine was well tolerated by all volunteers, but one person refused his second inoculation after having fever, malaise, and haemoptysis due to a coincident lung abscess which promptly responded to ampicillin and cloxacillin.

ELISA, prevaccination sera - Of the 90 pre-vaccination sera, 13 had titres of 20 - 200, and 6 had titres of ≥ 20 upon re-titration. Of the six, 5 were in the range 24 - 43, and one was > 500 on three separate occasions.

Antibody responses - Rabies IgG antibody was found in all 87 sera that were obtained 14 days after the first injection. The range of the antibody responses was wide (1/43 to 1/9353) but 80 to 100% of the results differed by less than fourfold from the geometric mean titres for each group (Figure 10.10). The titres obtained in all three centres were similar, with GMTs for IM being higher than that for ID vaccination. Overall, the GMT was 1/1451 for ID vaccination and 1/1762 for the
Fig. 10.10 Geometric mean rabies IgG antibody titres after intradermal and intramuscular vaccination with HDCSV at Peshawar, Hyderabad, and Islamabad.

□ = Peshawar, ○ = Islamabad, △ = Hyderabad.
IM route, the difference not being significant.

A second injection, on day 28, resulted in a substantial rise in rabies antibody in most subjects a fortnight later. Day 42 titres again showed no significant difference between the three centres in antibody responses obtained by inoculations by either route, but at each centre titres were higher with the IM than with ID vaccination (Figure 10.9); the difference between the overall GMT of $1/9264$ for ID vaccination and $1/14075$ for IM vaccination was significant ($p<0.01$).

Reproducibility of ELISA - Of 31 titrations of the positive control serum included on each plate, 26 (84%) differed by less than a two-fold from the GMT ($1/1996$), and all differed by less than a four-fold. Repeat titrations of the positive standard and 14 test sera did not show any significant difference between the results obtained in Pakistan or England.

Laboratory tests of vaccine potency - According to two of the three laboratory tests applied there was no difference between the antigenicity of vaccines returned from each centre (Table 10.26). The NIH and SRD tests showed that the potency of vaccine returned from Pakistan closely resembled that of vaccine tested before the study began and vaccine held in London and then re-tested upon completion of the project. However, potency testing by ELISA suggested that all three batches returned from Pakistan had a significant loss of potency, with vaccine from Hyderabad faring the worst.
Table 10.26  Study 11; Results of the laboratory potency before and after the field-test in man.

<table>
<thead>
<tr>
<th>Test</th>
<th>Vaccine potency before field testing</th>
<th>&quot;Control&quot; held in London</th>
<th>&quot;Test vaccine&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vaccine potency after field testing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIH antigenic value (IU)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.1</td>
<td>2.0</td>
<td>3.5</td>
</tr>
<tr>
<td>SRD (IU)</td>
<td>(6.9 - 8)</td>
<td>(7.4 - 8.7)</td>
<td>7.5</td>
</tr>
<tr>
<td>(95% confidence limits in parentheses)</td>
<td>(6.5 - 7.8)</td>
<td>(7.1 - 8.4)</td>
<td>(6.8 - 8.1)</td>
</tr>
<tr>
<td>Antibody production test (mean vaccine dilution giving antibody titre 1/100)</td>
<td>1020&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1085&lt;sup&gt;b&lt;/sup&gt;</td>
<td>709&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

NOTE: a - Mean of 8 tests; b - mean of 4 tests; c - significant loss of potency compared with potency of vaccine before dispatch for field tests (p<0.05).

30. Study 11 - Discussion

Potency testing of vaccines that are returned from centres to which they have been distributed is an important but often overlooked investigation. Many reports from Europe and North America testify to the excellent antibody response and protection of persons immunised with HDCSV, but until this study was undertaken none had adequately attempted to assess its potency and application in rural areas in developing countries. Lyophilised HDCSV stored at +4°C has a much longer shelf-
life than NTV and can be kept at 37°C for one month without loss of potency in mice (Nicholas et al, 1978, Chippaux et al, 1985). Accordingly, it was considered that HDCSV might be sufficiently thermostable to contend with the fluctuating, high ambient temperatures that it may encounter during transit to, or storage in, remote regions of the tropics. The data reported strongly support this assumption (Nicholson et al, 1983).

Experience gained in Pakistan and other developing countries indicates that much rabies vaccine is used within a month or so of its arrival at a treatment centre because demand is so great. We ensured, therefore, that batch A be sent the relatively short distance of 110 miles to Peshawar by the best available means, and that it be stored locally in a refrigerator for only three weeks before use. Hence the antibody response at this centre should represent that best that could be expected at a provincial clinic anywhere in the world. By contrast, the first injection with batch B vaccine was given after 7 weeks' exposure to temperatures of 26-40°C, and the second after a further 4 weeks' exposure to similar ambient temperatures. Although such exposure to high temperatures is considered to be a critical test of vaccine stability, the possibility that some rabies vaccines are treated more harshly cannot, of course, be totally discounted. I chose day 14 for the first antibody titration because it would be likely to show up important differences between the early responses to vaccination. Similarly blood samples were taken 14 days after the second injection to show whether a 'secondary' response could compensate for any possible loss of vaccine potency.

Despite the high temperatures to which it was exposed in Hyderabad, the vaccine evoked in all subjects substantial antibody responses, similar to those obtained in Peshawar and Islamabad, where it was stored under much better conditions. The response to small (0.1ml) doses of vaccine (given ID) could be said to be a more strenuous test of stability than the response to much larger doses of 1.0ml (given IM or SC) of vaccine stored under similar conditions. It is notable, therefore, that the response to the first IM dose was indistinguishable from that to
the first ID dose. However, by 14 days after the second injection the GMTs were 1.1 to 1.8 times higher with the IM route; the overall 1.5-fold difference was statistically significant. Rather than indicate a subtle loss of the vaccine's potency, the results are, in fact, in complete agreement with those reported previously that, despite induction of lower antibody responses, the intradermal route is both effective and economic (Turner et al, 1982).

Laboratory findings that vaccines returned from each centre did not differ between each other in potency are in accordance with the observations in man. Interestingly, the antibody-production test in mice suggested a slight loss of antigenicity for vaccine returned from each centre, the loss being greatest in batch B, from Hyderabad. However, no loss of potency was detected by the NIH and SRD tests, and since substantial titres of antibody developed in man and mice during the study, the loss of antigenicity seems trivial and clinically insignificant.

The thermostability of HDCSV in the tropics has been confirmed by more recent experience. In August 1983, the death of an American Peace Corps Volunteer (PCV) in Kenya after ID pre-exposure immunization with HDCSV (MMWR, 1983a) resulted in a field evaluation of pre-exposure use of HDCSV (MMWR, 1983b). The results indicated lower antibody titres of PCVs immunized ID in tropical countries as compared with those immunised in the United States and Europe, yet the potency of available vaccine used in the PCVs was re-evaluated and, with the exception of one batch, was found to be satisfactory. Subsequent studies revealed the immuno-suppressive activity of chloroquine. At an informal WHO meeting held in Annecy in June 1988, details were presented of 17 'treatment failures' within Third World countries despite post-exposure treatment with HDCSV or other potent tissue culture vaccines. Treatment flaws were noted in at least 15 cases. Interestingly, the potency of 6 lots of vaccine had been re-tested upon return from the field and each lot satisfied current WHO requirements, i.e., had retained a potency of > 2.5IU (Roumiantzeff, personal communication). Dr Roumiantzeff further stated that the
shelf-life of HDCSV at 4°C was at least 9 years and that tests of PVRV indicated that it was stable at 45°C for more than 1 year.

The study showed not only that lyophilized HDCSV is exceptionally thermostable but also that the ELISA technology for rabies antibody measurement was readily transferred to Pakistan, as the results in Islamabad and London were in extremely close agreement. It was noted, however, that 5 of 90 pre-vaccination sera had titres of 1/24 to 1/43 and a sixth had a titre of >1/500, which was confirmed by re-testing. This volunteer did not have an anamnestic response upon vaccination. Consideration should therefore be given to re-setting the boundary between antibody 'positivity' and 'negativity' at, say, 1/200, and/or the use of the 8 negative control sera to clearly distinguish non-specific binding from low titres of antibody.

31. Study 12 - Assessment of economical regimens of HDCSV for post-exposure prophylaxis

The effective antigenicity of HDCSV is enhanced by giving it ID instead of IM and by the use of aluminium hydroxide adjuvant (Kuwert et al, 1978c). To devise an efficient, economical regimen of HDCSV for a subsequent post-exposure study in Thailand, the effect of multisite ID inoculation, single-site ID inoculation, and adjuvanted SC vaccine were compared with a regimen of HDCSV equivalent to the first 5 doses of the regimen recommended by the WHO (i.e., the current US Public Health Service recommendation), and with Semple vaccine. Drs David and Mary Warrell, my principal collaborators in Thailand, provided coded sera which I titrated by ELISA blindly.

88 people were immunised with either HDCSV (Batch V0133, potency 2.55 U/ml) or 5% Semple vaccine (Thai Red Cross, Protective value 30 900 LD50 by the Habel test). The majority of vaccinees (49) were patients attending the Queen Saovabha Memorial Institute (Thai Red Cross Centre), Bangkok, these subjects all
had doubtful contact with animals suspected of being rabid, or occasionally with human rabies patients, sufficient to warrant vaccination according to the criteria of the Institute. The remaining 39 subjects were healthy volunteers from the staff of the Bangkok Hospital for Tropical Diseases. None of the subjects had had previous rabies vaccination. All patients and volunteers gave informed consent, and the study protocols were passed by the ethical committees of Northwick Park Hospital, Harrow, and Mahidol University, Bangkok.

The vaccinees were immunised as shown in Table 10.27. The injection site

Table 10.27 Study 12; Vaccination regimens of subjects.

<table>
<thead>
<tr>
<th>Vaccine, volume (ml)</th>
<th>Day given</th>
<th>No. of subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>route</td>
<td></td>
<td>Vaccine only</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>HDCS, 1.0</td>
<td>IM</td>
<td>0, 3, 7, 14, 28, (91)</td>
</tr>
<tr>
<td>HDCS, 0.1 x 8</td>
<td>ID</td>
<td>0</td>
</tr>
<tr>
<td>0.1 x 4</td>
<td>ID</td>
<td>14</td>
</tr>
<tr>
<td>0.1 x 1</td>
<td>ID</td>
<td>(91)</td>
</tr>
<tr>
<td>HDCS, 0.1 + Adj</td>
<td>SC</td>
<td>0, 3, 7, 14, 28, (91)</td>
</tr>
<tr>
<td>HDCS, 0.1</td>
<td>ID</td>
<td>0, 3, 7, 14, 28, (91)</td>
</tr>
<tr>
<td>Semple 2.0</td>
<td>SC</td>
<td>0 to 13, 21, 28, 91</td>
</tr>
<tr>
<td>HDCS, 2.0</td>
<td>IM</td>
<td>0, 3</td>
</tr>
<tr>
<td>1.0</td>
<td>IM</td>
<td>14</td>
</tr>
<tr>
<td>1.0</td>
<td>IM</td>
<td>91</td>
</tr>
</tbody>
</table>

for single dose regimens was the left deltoid area; the ones used for the 4-site injections were the left and right deltoid and thigh areas; and for 8-site injections they were the left and right deltoid, suprascapular, thigh, and lower abdominal wall areas. These sites were chosen to involve a maximum number of groups of lymph nodes. Each dose of adjuvanted vaccine comprised 1.0-ml of saline containing 0.1ml
of HDCSV and 0.98 mg of aluminium hydroxide; the resultant suspension was held at +4°C for several hours before administration. Semple vaccine was given in 2 ml volumes into the lower abdominal wall on 14 consecutive days, with booster doses on days 21, 28, and 91; this booster regimen approximates the one in use at the Queen Saovabha Memorial Institute, but to simplify follow-up, booster doses were given on the same day of the week. HRIG (Batch GR4) was given in a single dose of approximately 40 IU/kg IM, into the buttock to randomly selected subjects at the time of the first injection. Blood samples were taken on days 0, 7, 14, 28, 91, and 182. Rabies IgG antibody was measured by ELISA as described previously. Eight sera were individually used as negative controls, and a titre of ≥1 in 20 was taken as positive. Rabies neutralising antibody was measured by the rapid immunofluorescence focus inhibition test (RFFIT) by Dr Xueref in France. Titres of <0.5 IU/ml were not recorded. Statistical comparisons were made using Fisher's exact and an unpaired t test with log_{10} values of the RFFIT titres in IU/ml and reciprocal titres obtained by ELISA. Statistical analysis of the day 182 results was not possible since the groups of 5 subjects were too small for comparison.

32. Study 12 - Adverse effects of vaccination

The subjects given HDCSV had mild symptoms and signs, and ID vaccination produced more local pruritus, but less pain than did the other methods. The recipients of adjuvanted vaccine had notably few signs of reaction and 5 of the 16 recipients had no symptoms throughout the entire course of vaccination. In contrast, most patients receiving Semple vaccine had painful injection sites with local lymphadenopathy and frequent systemic symptoms. One patient who had an especially severe local reaction developed paraesthesiae, limb pains and weakness on day 14; there were no objective neurological signs, but the clinical impression was one of a neurological reaction to vaccination. All symptoms resolved within two weeks.
33. Study 12 - Serological results

Antibody prevalence on day 0. Two of the 88 Thai sera had pre-vaccination titres of 1 in 476 and 1 in 928; repeat testing confirmed the high OD readings, and sera collected from the same individuals on day 7 were similarly positive. None of the remaining day 0 sera had titres of \(1 \times 10^{-20}\). All of the day 0 sera had rabies neutralising antibody titres of < 0.5 IU/ml.

Antibody prevalence on day 7. IgG rabies antibody was detected in 4 of 10 subjects given the 8-site ID regimen, in 4 of 7 given the 2-site IM regimen, but in one or none of the subjects in the other groups. With the exception of the comparison between 2-site IM regimen (4 of 7) and the group given adjuvanted vaccine (0 of 10), the differences were not statistically significant. The 8-site ID regimen was the only group in which rabies neutralising antibody was detected on day 7, in 2/10 subjects.

IgG antibody response to vaccine without HRIG. By day 14, the recipients of the WHO IM regimen all had titres \(\geq 1 \times 10^{4}\) and the GMT of 1 in 16 155 was significantly higher than any other regimen, except the 8-site ID regimen and the 2-site IM regimen. The WHO IM regimen had the highest GMTs on every occasion, but they were not significantly greater than the 8-site ID regimen until day 91 (Table 10.28). The 8-site ID regimen gave a GMT of 1 in 14 206 on day 14 which was significantly greater than the remaining regimens, except for the 2-site IM regimen. On day 14, the adjuvant regimen, the 1-site ID regimen, and the Semple vaccine evoked statistically indistinguishable responses with GMTs of 1 in 1 625, 1 in 4 609, and 1 in 7 114 respectively. In general, the titres induced by all regimens were well maintained even without day 91 booster; thus, from their peak on day 14 or 28, they declined by an average of 3.5 and 3.6-fold by days 91 and 182 respectively. However, occasional sera, notably those from the adjuvant and 1-site ID groups, had day 91 titres of less than 1 in 2 000. Nonetheless, substantial titres were still present on day 182 provided that a booster was given on day 91.
Table 10.28. Geometric mean and range of IgG rabies antibody responses to HDCSV and Semple vaccine

<table>
<thead>
<tr>
<th>Vaccine, volume (ml)</th>
<th>Days after first vaccination</th>
<th>No boost</th>
<th>Booster</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>route (1st dose)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaccine only groups:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDCS, 1.0</td>
<td>IM</td>
<td>GMT</td>
<td>range</td>
</tr>
<tr>
<td>HDCS, 0.1 x 8</td>
<td>ID</td>
<td>GMT</td>
<td>range</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1-9)</td>
</tr>
<tr>
<td>HDCS, 0.1 + Adj SC</td>
<td>GMT</td>
<td>range</td>
<td>1.62</td>
</tr>
<tr>
<td></td>
<td>(1-7)</td>
<td>(1-8)</td>
<td>(300-13 193)</td>
</tr>
<tr>
<td>HDCS, 0.1</td>
<td>ID</td>
<td>GMT</td>
<td>range</td>
</tr>
<tr>
<td>Semple, 2.0</td>
<td>SC</td>
<td>GMT</td>
<td>range</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1-18)</td>
</tr>
<tr>
<td>HDCS, 2.0</td>
<td>IM</td>
<td>GMT</td>
<td>range</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1-6)</td>
</tr>
</tbody>
</table>
Table 10.29. Geometric mean and range of IgG rabies antibody responses to HDCSV and Semple vaccine: HRIG was administered with the first dose.

<table>
<thead>
<tr>
<th>Vaccine, volume/ml</th>
<th>0</th>
<th>7</th>
<th>14</th>
<th>28</th>
<th>90</th>
<th>No boost</th>
<th>182</th>
<th>Booster</th>
</tr>
</thead>
<tbody>
<tr>
<td>route (1st dose)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDCS, 1.0 IM GMT</td>
<td>1.4</td>
<td>13.6</td>
<td>19.779</td>
<td>14.446</td>
<td>11.130</td>
<td>9.329</td>
<td>4.972</td>
<td></td>
</tr>
<tr>
<td>HDCS, 0.1 x 8 ID GMT</td>
<td>1</td>
<td>17</td>
<td>8.915</td>
<td>6.244</td>
<td>2.411</td>
<td>2.889</td>
<td>4.064</td>
<td></td>
</tr>
<tr>
<td>HDCS, 0.1 + Adj SC GMT</td>
<td>1.7</td>
<td>7.7</td>
<td>13.59</td>
<td>7.308</td>
<td>3.781</td>
<td>1.527</td>
<td>6.693</td>
<td></td>
</tr>
<tr>
<td>HDCS, 0.1 ID GMT</td>
<td>1.2</td>
<td>7.6</td>
<td>6.589</td>
<td>4.661</td>
<td>4.737</td>
<td>1.701</td>
<td>3.641</td>
<td></td>
</tr>
<tr>
<td>Semple 2.0 SC GMT</td>
<td>1</td>
<td>19.9</td>
<td>7.122</td>
<td>4.129</td>
<td>2.373</td>
<td>-</td>
<td>2.795</td>
<td></td>
</tr>
<tr>
<td>range</td>
<td>(1)</td>
<td>(1-366)</td>
<td>(79.3-184.66)</td>
<td>(10.55-16.273)</td>
<td>(5.17-5.368)</td>
<td>-</td>
<td>(14.46-46.56)</td>
<td></td>
</tr>
</tbody>
</table>
IgG antibody response to vaccine with HRIG. Analysis showed that there was no significant difference between the antibody profiles of persons receiving vaccine only or vaccine plus HRIG for any of the schedules tested. However, there was a trend towards immunosuppression by passively administered HRIG for the multisite ID regimen on days 14 [GMTs of 14 206 (without HRIG) and 8 915 (with HRIG)] and 28 [GMTs of 11 968 (without HRIG) and 6 244 (with HRIG)]. The WHO IM group again had the highest GMTs, but because of the relatively small numbers of sera tested, comparisons with the other regimens mostly revealed non-significant differences (Table 10.29).

Neutralising antibody response to vaccine without HRIG (Table 10.30).

WHO IM regimen. - Although no subject had an antibody titre >0.5 IU/ml on day 7, by day 14 all had titres ≥ 16 IU/ml, and the GMT of 42.8 was significantly higher than that with any other regimen (p ≤0.033). This group had the highest GMTs on every occasion except for day 7.

8-site ID regimen. - On day 14, the GMT of 24.1 IU/ml was significantly higher than that in the adjuvant group (p = 0.006) and in the single site ID group (p = 0.007). On day 28 the GMT was similar to that of the IM group, but on day 91 it was lower than the IM group (p = 0.008). In the group not given a booster on day 91, one patient had a titre of <0.5 IU/ml on day 182.

Adjuvant regimen. - On days 14 and 28, the GMTs were lower than with the 8-site ID regimen (p = 0.006 and 0.007 respectively), but similar to those in the single-site ID group. By day 91, the GMT did not differ from the WHO IM regimen of the 8-site ID group. When no booster was given on day 91, the titres varied widely on day 182, and 1 patient had a titre of < 0.5 IU/ml. Nevertheless, the GMTs on that day were next highest after the IM group whether or not a booster was given.

1-site regimen. - Among those receiving HDCSV, this group had the slowest response. 1 patient had a titre of < 0.5 IU/ml on day 14. By day 91 the GMT was statistically similar to that of the 8-site ID group, but significantly lower than in the WHO IM group (p = 0.003) and adjuvant groups (p = 0.03). When no booster
Table 10.30. Geometric mean and range of rabies neutralising antibody responses to HDCSV and Semple vaccine

<table>
<thead>
<tr>
<th>Vaccine, volume (ml)</th>
<th>Vaccine route (1st dose)</th>
<th>GMT 0</th>
<th>GMT 7</th>
<th>GMT 14</th>
<th>GMT 28</th>
<th>GMT 90</th>
<th>GMT 182</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDCS, 1.0</td>
<td>IM</td>
<td>&lt;.5</td>
<td>&lt;.5</td>
<td>42.8</td>
<td>38.9</td>
<td>12.1</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>range</td>
<td>(&lt;.5)</td>
<td>(&lt;.5)</td>
<td>(16-91)</td>
<td>(17-82)</td>
<td>(3.7-59)</td>
<td>(2-5.4)</td>
</tr>
<tr>
<td>HDCS, 0.1 x 8</td>
<td>ID</td>
<td>&lt;.5</td>
<td>&lt;.5</td>
<td>24.1</td>
<td>27.3</td>
<td>4.1</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>range</td>
<td>(&lt;.5)</td>
<td>(&lt;.5-1.8)</td>
<td>(10-54)</td>
<td>(13-63)</td>
<td>(1-12)</td>
<td>(&lt;.5-2.7)</td>
</tr>
<tr>
<td>HDCS, 0.1 + Adj SC</td>
<td>GMT range</td>
<td>&lt;.5</td>
<td>&lt;.5</td>
<td>5.6</td>
<td>12.9</td>
<td>5.6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>(&lt;.5)</td>
<td>(&lt;.5)</td>
<td>(1.4-23)</td>
<td>(5.8-35)</td>
<td>(1.3-18)</td>
<td>(1-12)</td>
<td>(1-12)</td>
</tr>
<tr>
<td>HDCS, 0.1</td>
<td>ID</td>
<td>&lt;.5</td>
<td>&lt;.5</td>
<td>6.0</td>
<td>11.3</td>
<td>2.5</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>range</td>
<td>(&lt;.5)</td>
<td>(&lt;.5)</td>
<td>(&lt;.5-23)</td>
<td>(3.2-18)</td>
<td>(0.7-6.4)</td>
<td>(&lt;.5-0.9)</td>
</tr>
<tr>
<td>Semple 2.0</td>
<td>SC</td>
<td>&lt;.5</td>
<td>&lt;.5</td>
<td>1.9</td>
<td>3.8</td>
<td>1.1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>range</td>
<td>(&lt;.5)</td>
<td>(&lt;.5-15)</td>
<td>(&lt;.5-21)</td>
<td>(0.5-3.4)</td>
<td>-</td>
<td>(&lt;.5-2.5)</td>
</tr>
<tr>
<td>HDCS, 2.0</td>
<td>IM</td>
<td>&lt;.5</td>
<td>&lt;.5</td>
<td>16.8</td>
<td>17.7</td>
<td>1.4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>range</td>
<td>(&lt;.5)</td>
<td>(&lt;.5-15)</td>
<td>(9.6-33)</td>
<td>(9.6-33)</td>
<td>(0.7-3.1)</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 10.31. Geometric mean and range of rabies neutralising antibody responses to HDCSV and Semple vaccine: HRIG was administered with the first dose.

<table>
<thead>
<tr>
<th>Vaccine, volume (ml)</th>
<th>route (1st dose)</th>
<th>Days after first vaccination</th>
<th></th>
<th></th>
<th></th>
<th>No boost</th>
<th>Booster</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>7</td>
<td>14</td>
<td>28</td>
<td>90</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Vaccine plus HRIG:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDCSV, 1.0</td>
<td>IM</td>
<td>GMT range</td>
<td>&lt;.5</td>
<td>&lt;.5</td>
<td>28.8</td>
<td>43.5</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(.5)</td>
<td>(.5)</td>
<td>(7-69)</td>
<td>(8.1-60)</td>
<td>(2.6-19)</td>
<td>(3-12)</td>
</tr>
<tr>
<td>HDCSV, 0.1 x 8</td>
<td>ID</td>
<td>GMT range</td>
<td>&lt;.5</td>
<td>&lt;.5</td>
<td>3.6</td>
<td>10.8</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(.5)</td>
<td>(.5)</td>
<td>(5.2-20)</td>
<td>(0.5-2.7)</td>
<td>(2-3.6)</td>
<td>(3-10)</td>
</tr>
<tr>
<td>HDCSV, 0.1 + Adj SC</td>
<td>ID</td>
<td>GMT range</td>
<td>&lt;.5</td>
<td>&lt;.5</td>
<td>.74</td>
<td>14.4</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(.5)</td>
<td>(.5)</td>
<td>(5.1-37)</td>
<td>(0.8-3.1)</td>
<td>(3-5.1)</td>
<td>(1.8-2.3)</td>
</tr>
<tr>
<td>HDCSV, 0.1</td>
<td>ID</td>
<td>GMT range</td>
<td>&lt;.5</td>
<td>&lt;.5</td>
<td>4.5</td>
<td>18.5</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(.5)</td>
<td>(.5)</td>
<td>(8.3-194)</td>
<td>(0.5-38)</td>
<td>(3-5.1)</td>
<td>(1.8-3.1)</td>
</tr>
<tr>
<td>Semple 2.0</td>
<td>SC</td>
<td>GMT range</td>
<td>&lt;.5</td>
<td>&lt;.5</td>
<td>&lt;.5</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(.5)</td>
<td>(.5)</td>
<td>(5-2.7)</td>
<td>(0.5-0.96)</td>
<td>(5-0.96)</td>
<td>(5-0.73)</td>
</tr>
</tbody>
</table>
was given on day 91, 3 of 5 subjects had titres of <0.5 IU/ml on day 182.

**Semple regimen.** - Unlike the ELISA IgG results, this group had the lowest GMTs on every occasion. On day 14, 2 patients had titres <0.5 IU/ml although there was no significant difference from the adjuvant and single-site ID groups. On days 28 and 91 the GMTs were lower than those of all the other regimens (p ≤0.019 and p ≤0.014 respectively). On day 182, 5 patients had titres <0.5 IU/ml.

**2-site IM regimen.** - The GMTs of this group were lower than those of the 8-site ID group on every occasion.

**Neutralising antibody response to vaccine with HRIG (Table 10.31).** - The IM group was the only one in which all the subjects had titres >0.5 IU/ml from day 14 onwards and in which there was no evidence of immunosuppression by the administered antibody. On day 14, titres of <0.5 IU/ml were found in one person in the 8-site ID group; 3 in the adjuvant group; 1 in the single-site ID group; and 5 in the Semple group. The GMTs were depressed by HRIG on days 14, 28, and 91 (p<0.01) in the 8-site ID group and on day 14 in the Semple group (p = 0.008). The maximum GMT after Semple vaccination was 0.6 IU/ml.

3.4. **Study 12 - Discussion**

This study (Warrell et al, 1983) compares the IgG and neutralising antibody responses of economical "candidate" post-exposure regimens for HDCSV with a schedule equivalent to the first 5 doses of the regimen approved by WHO, and a regimen of Semple vaccine used presently in Thailand following mild exposures. The highest IgG and neutralising antibody titres were found in response to the WHO IM regimen although the 8-site ID regimen, which requires only one-quarter of the volume of vaccine, gave comparable results. The single site ID regimen and adjuvanted vaccine regimen evoked antibody responses in all cases although at much lower levels than with the other HDCSV regimens.

The ID regimen showed many features of an ideal vaccination schedule. Neutralising antibody was detected earlier than in any other group. The antibody
titres were consistently high, as high as that with the IM regimen on day 28, and always >0.5 IU/ml provided that a booster dose was given on day 91. The side-effects were negligible and although ID injections are more difficult to give than IM or SC ones, the use of 8 sites allows a margin of safety, as erroneous SC injections at one or two sites are unlikely to impair the antigenic stimulus.

The dose of HRIG recommended by WHO is 20 IU/kg, but it has been suggested that a larger dose may give better protection during the first week after infection, although decreasing the effectiveness of the vaccine (Turner, 1981; Mertz et al, 1982). The dose of 40 IU/kg used in this study is therefore a severe test of low-dose vaccine regimens, and it considerably suppressed the high antibody titres on days 14, 28, and 91 in those given the 8-site ID regimen.

The data provided by the ELISA led to several modifications to the candidate multisite ID regimen prior to its evaluation in a postexposure trial in Thailand. Because HRIG evidently caused immunosuppression (later confirmed by the RFFIT), the second series of injections carried out on day 14 was brought forward to day 7. Also since the day 91 IgG titres were lower in response to the 8-site ID regimen than the WHO IM regimen, an additional 0.1ml booster was introduced on day 28 to maintain the consistently high titres comparable to the WHO IM regimen.

The response to adjuvanted HDCSV was similar to that obtained with the single-site ID regimen. Because SC injections are easier to give than ID ones, the use of multiple site adjuvanted vaccine may be advantageous. The 2-site IM regimen did not produce antibody earlier than the standard IM method, in contrast to the findings of Anderson et al (1981).

35. **Study 13 - Multisite intradermal and multisite subcutaneous vaccinations**

The results of study 12 led to an experimental regimen for postexposure prophylaxis which was assessed in patients bitten by rabid animals in parallel with this study.
During previous studies it was recognized that skill is required to give intradermal injections, particularly to restless children, and that a high dose of HRIG suppressed the humoral response. In this study, we halved the number of ID injection sites, used multisite subcutaneous injections, and adjusted the schedule of injections in an attempt to overcome these problems.

Volunteers were recruited at the Queen Saovabha Memorial Institute, Bangkok, during my stay in Thailand for Study 14. 98 patients (45 male, 53 female) who had doubtful contact with a rabid animal and were therefore at very low risk of rabies infection, but who would otherwise have received Semple vaccine, gave informed consent to participate in the study. No patient had previous contact with a rabid animal or had ever received rabies vaccine. They were randomly assigned to one of 12 vaccine regimens (Table 10.32) and were immunised with HDCSV Lot No.

Table 10.32  Study 13; Vaccination regimens of subjects.

<table>
<thead>
<tr>
<th>Regimena (no of sites)</th>
<th>n</th>
<th>Dose per site (number of sites)</th>
<th>Total dosesb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 0</td>
<td>Day 7</td>
</tr>
<tr>
<td>1 HDCSV id (8)</td>
<td>10</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>id (8)</td>
<td>id (4)</td>
</tr>
<tr>
<td>2 Adjuvanted HDCSV sc (8)</td>
<td>10</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sc (8)</td>
<td>sc (4)</td>
</tr>
<tr>
<td>3 HDCSV sc (4)</td>
<td>10</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sc (4)</td>
<td>sc (2)</td>
</tr>
<tr>
<td>4 HDCSV id (4)</td>
<td>10</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>id (4)</td>
<td>id (4)</td>
</tr>
<tr>
<td>5 Adjuvanted HDCSV sc (4)</td>
<td>10</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sc (4)</td>
<td>sc (4)</td>
</tr>
<tr>
<td>6 HDCSV id (8)</td>
<td>10</td>
<td>0.1 ml</td>
<td></td>
</tr>
<tr>
<td>(8) day 14</td>
<td></td>
<td>id (8)</td>
<td></td>
</tr>
</tbody>
</table>

a Regimens 7-12 = regimens 1-6 plus 40 IU/kg HRIG. Regimens 7 and 9 were given to 7 patients each, and regimens 8, 10, 11, and 12 to 6 patients each.

b Although each dose of Merieux HDCSV is normally 1ml when reconstituted, in practice a full dose is required for 8 x 0.1 or 4 x 0.2, and half a dose for 4 x 0.1 or 2 x 0.2 multisite vaccinations.

n = number of patients; id = intradermal; sc = subcutaneous
V1242 with a potency of 3.39 by the NIH test. Adjuvanted HDCSV was prepared by thoroughly mixing 0.1 ml HDCSV with 0.9 ml aluminium hydroxide suspension containing 0.98mg/ml. The solution was agitated vigourously immediately before injection to ensure suspension. The injection sites were located as in Study 12. HRIG (Batch GR4; potency 1148 IU/ml) was given in a single dose of approximately 40 IU/kg to randomly selected subjects at the time of their first vaccination.

Blood samples were collected on days 0, 7, 14, 28, and 91. Rabies neutralising antibody was measured by Dr Xueref in France and IgG antibodies were titrated by ELISA on return to Northwick Park Hospital. One patient did not attend for follow-up on day 14, and 4 did not attend on day 91, but these patients were in different treatment groups so the results were probably not significantly affected.

Day 0 sera were titrated by ELISA using the 8 negative control sera used in previous studies. Three pools of day 0 sera were then prepared, each pool contained approximately 30 sera and excluded any that were positive. Antibody titres were determined by calculating the intercept of the quadratic curve fitted to OD values of test sera, and the straight line fitted to the mean plus 2.5 standard deviations to the mean of OD values of the control pools. A titre of ≥1 in 200 was taken as positive. For the purpose of determining the GMTs, a titre of 1 in 40 was taken for titres of less than 1 in 200. Statistical comparisons were made using Fisher's exact test and an unpaired t test with log10 values of the reciprocal titre.

36. Study 13 - Adverse effects of vaccination

Local pain and itching and mild symptoms of "fever" and headache were commonly reported. Mild erythema, induration, and local adenopathy were seen. Patients were not examined daily, so an accurate assessment of the frequency of reactions was not possible. More of the patients receiving adjuvanted vaccine (19 of 32) than of the patients receiving aqueous vaccine intradermally (13 of 49) complained of pain at the injection sites (p = 0.0065). Altogether 27.5% of patients were free from
signs and symptoms throughout the study.

37. Study 13 - ELISA results

8-site ID regimens: the effect of moving the second series of injections from day 14 to day 7. Bringing forward the second series of injections from day 14 to day 7 resulted in a significant increase of day 14 titres from a GMT of 1 in 27,555 to 1 in 57,411 (Figure 10.11) which grew larger (from 1 in 9, 307 to 1 in 63, 878) when HRIG was also administered.

Comparison of 8- and 4-site ID and adjuvanted vaccine regimens. The administration of 8 injections instead of 4 on day 0 had a significant and beneficial effect on antibody responses (Figure 10.12). The effect was significant only on day 7 for the ID regimens when the GMTs were 1 in 52 and 1 in 362 for the 4- and 8-site schedules respectively (p = 0.015). On day 7, more of the subjects (6 of 10) in the 8-site ID group had IgG titres of ≥200 than in the 4-site ID group (1 of 10). The 8-site ID regimen also induced a significantly more rapid antibody response than the schedule of 8 adjuvanted injections. - on day 7, 0 of 9 in the 8-site adjuvanted vaccine group had titres of ≥ 200 (GMT 40) [p = 0.017 for a comparison of the titres of the 8-site ID and adjuvanted groups]. Accordingly it was not until days 14 and 28 that the GMTs of the 8-site adjuvanted group became significantly greater than those of the 4-site group (Figure 10.12).

Comparison of the antibody responses to a 4-site SC regimen, with an 8-site ID regimen. Apart from the 8-site ID group, the 4-dose SC group was the only one in which rabies IgG was detectable as early as day 7. As shown in Figure 10.13, the GMTs throughout the 91-day study period were indistinguishable from those of the 8-site ID regimen with the second dose given on day 7 (Group 1).

Effect of HRIG on active antibody responses. HRIG administered at a dose of 40 IU/Kg had no measurable suppressive effect on early antibody responses. Its effect on later responses are summarised in Table 10.33.
Fig. 10.11 GMTs following multisite ID regimens for HDCSV; evaluation of the response to second injections given either on day 7, or day 14 (the upper profiles follow day 7 injections).
Fig. 10.12 GMTs following multisite injections with HDCSV; comparison of the responses to 4, or 8 day 0 injections either administered ID (left hand panel), or SC (right hand panel) with aluminium hydroxide adjuvant (the upper profiles are those of the 8-site regimens).
Fig. 10.13 Comparison of GMTs raised by a 4-site SC regimen and an 8-site ID regimen for HDCSV.
Table 10.33  Study 13; Effect of passively administered HRIG (40 IU/kg) on actively induced rabies IgG.

<table>
<thead>
<tr>
<th>HDCSV volume (ml), route</th>
<th>No. of injections on given day</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>0.1 id</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>0.1 id</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>0.1 id</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>0.1 + Adj sc</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>0.1 + Adj sc</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>0.2 sc</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

38. Study 13 - RFFIT results

Regimens without HRIG. On day 7 antibody was detectable in all 30 patients given vaccine intradermally (groups 1, 4, and 6), but 16 of 30 patients in the groups given vaccine subcutaneously with or without adjuvant had none (Figure 10.14). The day 7 GMTs of the 8-site ID groups (1 and 6) and the 4-site ID group (4) were significantly higher than those of the two groups given adjuvanted vaccine (2 and 5) (p < 0.00015 and p < 0.005, respectively). If groups 1 and 6 were considered as a single group, the GMT was significantly higher than that of group 4 (p = 0.014).

By day 14 the antibody levels of all patients were between 4.1 and 126 IU/ml. The 8-site ID group given their second injections on day 14 (group 6) had the lowest GMT; it was significantly lower than those of groups 1, 2, and 4 (p < 0.008). The GMT of group 4 was higher than that of group 5 (p = 0.0015).

On day 28 the antibody levels ranged between 3.5 and 140 IU/ml. Group 2 had the highest GMT, significantly higher than those of groups 1 (p = 0.04), 3 (p = 0.02), and 5 (p = 0.05).

The range of antibody titres on day 91 was 1-35 IU/ml. There was no significant difference in the geometric mean titres of the groups (p > 0.07) with the
Fig. 10.14 Geometric mean titres (with 95% confidence limits) of antibody responses to HDCSV regimens without HRIG. Numbers at the base of some columns indicate number of patients with no detectable antibody.
Fig. 10.15 Geometric mean titres (with 95% confidence limits) of antibody response to HDCSV regimens with HRIG.
exception of group 6, which was significantly lower than group 2.

Regimens with HRIG. Antibody was detected in all patients on day 7 (Figure 10.15), but the GMTs of the 8-site ID groups (7 and 12) were lower than those of the groups receiving the corresponding regimens without HRIG (not significant; \( p = 0.35 \)). The GMTs of all the other groups were higher than those of the groups that did not receive HRIG, but the difference was significant only in the 4-site adjuvanted groups (group 11 versus group 5; \( p < 0.001 \)) and the 8-site adjuvanted groups (group 8 versus group 2; \( p = 0.037 \)).

On day 14 the GMTs were significantly lower than in all the groups that had received HRIG (\( p < 0.008 \)), except in group 11, than in the corresponding groups without HRIG. The biggest difference was the 15-fold difference between groups 6 and 12. One patient in group 12 had a titre of 0.2 IU/ml, the only one below 0.5 IU/ml.

On day 28 the antibody levels ranged from 1.2 to 29 IU/ml and the GMTs of all groups were similar. On day 91 the range of the antibody levels was 0.6 - 22 IU/ml. The GMT of group 8 was significantly higher than those of groups 9 and 12 (\( p \leq 0.009 \)). The results for the adjuvanted regimens were similar to those of group 7.

39. Study 13 - Discussion
In this study (Warrell et al, 1984) the RFFIT was evaluated to a more sensitive threshold level of 0.1 IU/ml instead of 0.5 IU/ml as used in Study 12. By contrast, the sensitivity of the ELISA decreased when the threshold level was increased prospectively to 1 in 200. On day 7, when neutralising antibody was detectable in everyone given multiple ID injections, IgG titres of $\geq 1$ in 200 were detected in only 10 of 20 people given 8 ID injections (1 and 6), 13 had titres of $\geq 1$ in 40, and 16 showed increases from $<1$ in 1 to $\geq 1$ in 10. Presumably the discrepancy is due to the RFFIT detecting the early IgM antibodies, which largely remain in the intact
circulation and are arguably of limited value in rabies.

Overall there was close agreement between the results of the ELISA and RFFIT. On day 7, both tests showed that the 8-site ID regimen had the highest GMTs which were significantly higher than those of the 4-site ID group (4) and the 8-site adjuvanted vaccine group (2). Both tests showed the GMTs of the 8-site ID group (1) and 4-site ID group (4) to be higher than those of the 2 groups given adjuvanted vaccine (2 and 5), though the IgG titres of the two 4-site groups (4 and 5; GMTs 32,212 and 24,682 respectively) were not significantly different. Both tests showed significantly higher day 14 titres in group 1 compared to group 6, and group 7 compared to group 12, showing that high titres were produced more rapidly by advancing the second injections from day 14 to day 7. As anticipated, this reduced the immunosuppression caused by passively-administered antibody.

Accurate intradermal injection is essential for effective use of reduced amounts of antigen but, unlike subcutaneous injection, it can be difficult to achieve, especially in young children and the elderly. This study shows that vaccine given intradermally at 8 sites reliably produces neutralising antibody within a week, whereas an identical volume of subcutaneous vaccine, with or without adjuvant, does not. Nonetheless, from day 14 onwards, all patients produced antibody titres above the arbitrary level of 0.5 IU/ml recommended by the WHO. The advantage of an extremely high antibody titre is uncertain, but in view of the frequent delays between exposure and post-exposure prophylaxis, and short-incubation period disease, it is highly probable that a rapid, high-titred response is of greater benefit than a slower but equally high-titred response, e.g., to the multisite adjuvanted regimen.
40. **Study 14 - Trial of multisite intradermal immunisation with HDCSV for post-exposure rabies prophylaxis**

The previous studies show that a safe and economical regimen, in which ~30% of the normal amount of HDCSV is given ID at multiple sites, rapidly produces high titres of neutralising and IgG antibodies. Moreover, a single-dose multisite ID regimen was shown to protect rabbits against challenge with street virus. This study compares a multisite ID regimen with the standard Semple vaccine regimens used in Thailand for patients bitten by rabid animals.

A total of 155 patients attending the Queen Saovabha Memorial Institute (QSMI), Bangkok, were recruited to the study after it had been confirmed by the fluorescent-antibody test that the brain of a dog or cat that had bitten them contained rabies antigen. The animals' teeth had penetrated the patients' skin within the previous 5 days in every case. All patients denied having previous rabies vaccine treatment and gave informed consent.

To comply with standard practice at QSMI, patients were divided into two groups according to the severity of exposure to the virus. Seventy patients bitten on the head, neck (above the clavicles), or hand, with multiple bites, deep bites, or lesions that required sutures were regarded as 'severe' cases and were randomly allocated to one of two regimens:

**HDCSV.** - Lot V1242 (Antigenic value, 3.39 U/ml) was given ID according to the following schedule. On day 0, 0.1 ml was given at 8 sites in the deltoid, suprascapular, thigh, and abdominal wall areas; on day 7, 0.1 ml was given at 4 sites in the deltoid and thigh regions and on days 28 and 91, 0.1 ml was given at one site in the deltoid.

**Semple vaccine** - Semple vaccine, consisting of 5% sheep brain suspension (Thai Red Cross, Lot 8 of potency 264 900 LD₅₀ and a few final booster doses of Lot 6 of potency 124 500 LD₅₀) was given in 5-ml doses on days 0-20 and again on days 28, 42, and 91.

All severe cases were also given equine antirabies serum (EARS) (Institut
Pasteur lot 55) on day 0; the dose was 40 IU/kg according to the package insert, but subsequent titration indicated that the potency was double, at 80IU/kg. Half was given intramuscularly and half was infiltrated around the wound when possible.

The remaining 85 patients with mild exposure were randomly allocated to one of two regimens; according to the standard practice at QMSI, mild cases were not given hyperimmune serum despite their exposure to animals proved to be rabid.

**HDCSV.** - Vaccine was given as for severe exposure but without EARS.

**Semple vaccine** - 2-ml of vaccine was given subcutaneously on days 0-13 and again on days 28, 42, and 91.

Patients were seen regularly for 1 year. Venous blood samples were taken on days 0, 7, 14, 28, and 91, and at 6 and 12 months. Serum neutralising antibody titres were measured blind by Dr Xueref on coded samples in Lyon by RFFIT and expressed in International units (IU). All sera were tested on days 0, 7, and 14, and the remaining samples up to one year were tested in 136 patients for whom complete sets were available. Geometric mean titres of the treatment groups were compared by a students t test with log10 values in IU. Antibody levels of <0.1 IU/ml were counted as 0.01 for the purpose of the calculation. Rabies IgG titres were measured at the Clinical Research Centre, Northwick Park Hospital in all specimens up to day 91, and in approximately half of the 6 month sera. Three pools of day 0 sera from study 13 were used as negative control sera. A titre of 1 in 200 was used as the threshold between antibody positivity and negativity. Geometric mean titres of the treatment groups were compared by a students t test with log10 values of the reciprocal titres. Antibody levels of 1 in < 200 were counted as 1 in 40 for the purpose of the calculation.

4.1. **Study 14 - Results**

Seventy-eight patients (36 severe and 42 mild exposure) were treated with HDCSV; 77 (34 severe and 43 mild exposure) were given Semple vaccine. Distribution of
the bites is shown in table 10.34. There were 66 women and 89 men. Mean age was 22.7 years (range 4-66), mean weight was 40.9kg (range 12-82), and the average delay between bite and start of vaccine treatment was 44.9 hours (range 3-138). These variables were comparable in the four treatment groups. In both types of exposure, the number of lesions inflicted and the proportion of patients who had washed the wounds immediately were similar for the Semple and HDCSV groups (Table 10.35). Among the severely exposed patients, 32 (46%) had been bitten at two or three different sites.

Table 10.34 Study 14; Distribution of bite-sites.

<table>
<thead>
<tr>
<th></th>
<th>Severe SV</th>
<th>Severe HDCSV</th>
<th>Mild SV</th>
<th>Mild HDCSV</th>
<th>Total Bites No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>34</td>
<td>36</td>
<td>43</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Head/neck</td>
<td>4</td>
<td>5</td>
<td>.</td>
<td>.</td>
<td>9 (5)</td>
</tr>
<tr>
<td>Arm</td>
<td>7</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>20 (10)</td>
</tr>
<tr>
<td>Hand</td>
<td>16</td>
<td>21</td>
<td>.</td>
<td>.</td>
<td>37 (19)</td>
</tr>
<tr>
<td>Trunk</td>
<td>7</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>16 (8)</td>
</tr>
<tr>
<td>Leg</td>
<td>15</td>
<td>15</td>
<td>24</td>
<td>24</td>
<td>78 (41)</td>
</tr>
<tr>
<td>Ankle/foot</td>
<td>4</td>
<td>7</td>
<td>11</td>
<td>11</td>
<td>33 (17)</td>
</tr>
<tr>
<td>Total bites</td>
<td>53</td>
<td>55</td>
<td>43</td>
<td>42</td>
<td>193 (100)</td>
</tr>
</tbody>
</table>

Table 10.35 Study 14; Details of animals and bites.

<table>
<thead>
<tr>
<th></th>
<th>Severe SV</th>
<th>Severe HDCSV</th>
<th>Mild SV</th>
<th>Mild HDCSV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biting species</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog</td>
<td>34</td>
<td>36</td>
<td>41</td>
<td>40</td>
</tr>
<tr>
<td>Cat</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Number of skin punctures</td>
<td>6.44 (1-31)*</td>
<td>5.63 (1-20)</td>
<td>2.51 (1-11)</td>
<td>2.57 (1-7)</td>
</tr>
<tr>
<td>Wound washed immediately (%)</td>
<td>62</td>
<td>69</td>
<td>49</td>
<td>31</td>
</tr>
<tr>
<td>Time between bite and treatment (h)</td>
<td>49.5 (6-120)*</td>
<td>37.1 (4.5-116)</td>
<td>46.4 (4.5-126.5)</td>
<td>46.4 (3-138)</td>
</tr>
</tbody>
</table>

* Range
Outcome - 149 (96%) patients were alive and well 1 year after their bite. Two (1.5%) patients died of unrelated causes; 1 man (mild SV group) who was a drug addict died of a heroin overdose 7 months after being bitten; 1 woman (severe SV) died of cervical carcinoma with renal failure 11 months after the bite. Four patients (3 SV, 1 HDCSV) moved away from Bangkok and were lost to follow-up after 6 weeks to 6 months of starting vaccination; all were in the mild exposure group. After two years, 11 patients (7%) had moved away but there was no suggestion that they had died from rabies. The remaining patients were alive and well.

Serological response to HDCSV - HDCSV consistently induced higher neutralising antibody titres than Semple vaccine (Figure 10.16). The HDCSV 'mild' group also had consistently higher IgG titres than the Semple 'mild' group, but, in contrast, from day 28 onwards, the Semple 'severe' group had higher IgG titres than the HDCSV 'severe' group (Table 10.36).

On day 0, two sera gave results of 0.2 IU/ml, but subsequent titres were below the group GMT on every occasion but one at 6 months after vaccination. The corresponding IgG antirabies titres were 1 in 23 and 1 in 5, and this inhibition was therefore regarded as non-specific and not the result of previous vaccination. Only one person had an IgG titre of 1 in ≥200 (1 in 5 959). The titre on day 7 did not rise significantly, but on day 14 it was 6-fold greater than the GMT.

Patients given HDCSV alone (HDCSV 'mild') produced significantly more neutralising and IgG antibody than with any other regimen on every occasion. On day 7, all but 5 (12%) of 42 patients had detectable neutralising antibody (Table 10.37). Significantly more of this group (15/42, 36%) had detectable IgG antibody than any other group (Table 10.38). The peak neutralising and IgG antibody titres of the HDCSV 'mild' group were both reached on day 14, in contrast to days 28 (neutralising antibody) and 42 (IgG antibody) for recipients of other regimens. From day 14 to 6 months after vaccination, the lowest values recorded were 1.5 IU/ml and 1 in 1 232. At 1 year, all results were ≥0.6 IU/ml with one exception of 0.2 IU/ml.
Fig. 10.16 Study 14. Geometric mean titres (with 95% confidence limits) of antibody response to rabies vaccine regimens.
Table 10.36. Study 14. Geometric mean and range of rabies IgG responses to HDCSV and Semple vaccine with and without EARS

<table>
<thead>
<tr>
<th>Regimen</th>
<th>GMT</th>
<th>Days after first vaccination</th>
<th>Days after first vaccination</th>
<th>Days after first vaccination</th>
<th>Days after first vaccination</th>
<th>Days after first vaccination</th>
<th>Days after first vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>7</td>
<td>14</td>
<td>28</td>
<td>42</td>
<td>91</td>
<td>6M</td>
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<tr>
<td><strong>Vaccine only groups:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDCS 'Mild'</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GMT range</td>
<td>(&lt;1-5959)</td>
<td>(&lt;1-73360)</td>
<td>(9095-375020)</td>
<td>(14123-136264)</td>
<td>(9135-134143)</td>
<td>(3930-181643)</td>
<td>(1232-34246)</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>144</td>
<td>54010</td>
<td>44681</td>
<td>44368</td>
<td>12153</td>
<td>5209</td>
</tr>
<tr>
<td>Semple 'Mild'</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GMT range</td>
<td>(&lt;1-119)</td>
<td>(&lt;1-409)</td>
<td>(128-33147)</td>
<td>(1646-404740)</td>
<td>(1269-2218618)</td>
<td>(255-16195)</td>
<td>(130-5849)</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>42</td>
<td>2918</td>
<td>12863</td>
<td>17498</td>
<td>5509</td>
<td>1630</td>
</tr>
<tr>
<td><strong>Vaccine plus EARS:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDCS 'Severe'</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GMT range</td>
<td>(&lt;1-119)</td>
<td>(&lt;1-767)</td>
<td>(879-60895)</td>
<td>(1549-50346)</td>
<td>(4120-76430)</td>
<td>(575-56361)</td>
<td>(396-22513)</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>52</td>
<td>7316</td>
<td>16236</td>
<td>26195</td>
<td>8507</td>
<td>3043</td>
</tr>
<tr>
<td>Semple 'Severe'</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GMT range</td>
<td>(&lt;1-93)</td>
<td>(&lt;1-57963)</td>
<td>(32-22182)</td>
<td>(3277-82187)</td>
<td>(2558-224313)</td>
<td>(150-61029)</td>
<td>(192-29409)</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>46</td>
<td>2603</td>
<td>23555</td>
<td>30710</td>
<td>8731</td>
<td>2053</td>
</tr>
</tbody>
</table>
Table 10.37  Study 14; Percentage of patients without detectable neutralizing antibody*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number sera tested</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 28</th>
<th>Day 91</th>
<th>6 mo</th>
<th>1 yr</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDCSV</td>
<td>42/39</td>
<td>98</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HDCSV + EARS</td>
<td>36/33</td>
<td>97</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SV 2ml</td>
<td>43/35</td>
<td>100</td>
<td>98</td>
<td>14</td>
<td>3</td>
<td>3</td>
<td>23</td>
<td>63</td>
</tr>
<tr>
<td>SV 5ml + EARS</td>
<td>34/29</td>
<td>100</td>
<td>68</td>
<td>68</td>
<td>3</td>
<td>3</td>
<td>20²</td>
<td>31</td>
</tr>
</tbody>
</table>

*Percentage to nearest whole number; antibody level <0.1 IU/ml

1 Number tested up to day 14/number tested from day 28 onwards

2 One extra sample tested

Table 10.38  Study 14; Percentage of patients without detectable IgG antibody*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number sera tested</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 28</th>
<th>Day 91</th>
<th>6 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDCSV</td>
<td>42</td>
<td>64</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HDCSV + EARS</td>
<td>36</td>
<td>88</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>SV 2ml</td>
<td>43</td>
<td>98</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>SV 5ml + EARS</td>
<td>34</td>
<td>96</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Percentage to nearest whole number; antibody level <1 in 200

EARS significantly suppressed the GMT of both the IgG responses to ID HDCSV on days 7-42 and the neutralising antibody responses on every occasion tested. Maximal suppression of the neutralising and IgG antibody responses occurred on day 14, when the GMTs were reduced 18- and 7.6-fold respectively. On day 7, 33 of 36 (91.7%) patients given HDCSV and EARS had neutralising antibody, but only 2 had levels of >0.5 IU/ml, and only 4 of 36 had rabies IgG (human) antibody. From day 14 onwards, rabies neutralising and IgG antibodies were found in all sera (tables
10.37 and 10.38). On day 28, 1 of 33 patients had not yet attained a level of 0.5 IU/ml and another had fallen below that level since day 14. The GMT of 4.26 IU/ml on day 28 was statistically similar to the 2.8 IU/ml produced by the SV 'severe' group. The IgG titres of the HDCSV and SV 'severe' groups were statistically similar on all days up to 6 months except day 14, when the GMT of the HDCSV group was several-fold higher. On day 91, at 6 months, and at one year the lowest levels recorded in the HDCSV 'severe' group were 0.7, 0.4, and 0.3 IU/ml respectively.

*Serological response to Semple vaccine* - Among patients who had Semple vaccine alone, only 1 of 43 had neutralising antibody (0.1 IU/ml) on day 7. Another had no neutralising antibody at any stage of the study, but he did develop an IgG response by ELISA. In 7 of 35 patients (20%), all antibody levels were <0.5 IU/ml. One year after vaccination had begun, 63% had no detectable neutralising antibody.

Eleven of 34 (32%) given SV and EARS had neutralising antibody on day 7, but the number did not increase by day 14. Interestingly, the ELISA detected rabies IgG antibodies in 2 of 34 patients on day 7, and in 30 of 33 on day 14. On day 14, the geometric mean neutralising antibody titre of the Semple 'severe' group was 0.02 IU/ml, one tenth of that in the 'mild' group. Thus on day 14, suppression by the high dose of EARS was obvious (Figure 10.16), but cannot be quantified because the 'severe' group received a much greater volume of vaccine. In contrast the IgG titres of the 'severe' and 'mild' groups were statistically similar. One patient had no detectable neutralising antibody at any time and in another the titre never reached 0.5 IU/ml. On day 28 the GM neutralising and IgG titres were similar to those of the HDCSV severe group.

*Adverse effects* - Side effects were generally much more troublesome with SV than HDCSV. 29% of all HDCSV recipients had no signs or symptoms related to the vaccine throughout the study in contrast to only 6% who had SV (p <0.0005). Pain, erythema, and induration at the injection site occurred more frequently after SV than HDCSV (70 versus 33%, 55 versus 1%, and 36 versus 6%, respectively; for all comparisons p <0.0005). Two patients given HDCSV alone had an urticarial rash
on their limbs on day 28 and 36, accompanied by fever in one case. Two SV recipients complained of numbness of hands (on day 7) or legs (on day 42), but no objective signs were elicited and the symptom resolved within three days. EARS was generally well tolerated. One patient given EARS complained of buttock pain, but 2 manifested erythematous lesions at the sites of infiltration on days 7 and 8 while another (given HDCSV) had an urticarial maculo-papular rash on the legs on day 7.

42. Study 14 - Discussion

These data (Warrell et al, 1985) show that Semple vaccine and the economical multisite schedule of immunisation with HDCSV are both effective for postexposure rabies prophylaxis. All 155 patients had penetrating skin lesions by animals shown to be rabid, none died from rabies encephalitis, and therefore there was no difference between the different treatment regimens.

The degree of protection is difficult to assess since the risk of developing rabies after a bite by a rabid animal is uncertain. Mortality rates of 35-71% have been recorded when the infectivity of the biting animal is established by the death of another person or animal (Semple 1919; Cornwall, 1923; Nikolic, 1952; Gremliza, 1953; Veeraraghavan, 1969). Observations made in the era before vaccination indicate considerable variation in human mortality after dog and cat bites ranging from 2.5% to 44%, depending upon the site and severity of the bites (Babes, 1912). Most studies cited by Babes assess the mortality at approximately 15%, but none of the observations were made when rabies could be confirmed in the biting animal, so they may have produced an underestimate. On the basis of these data, an estimated 15% (23 of 155) of the patients in the current study would have died had they remained untreated.

Using Gross & Bearman's (1985) figures for the maximum effectiveness of rabies vaccination, which assumes a 15% incidence of rabies in non-vaccinated subjects, the proven effectiveness of treatment in this study, with 99% confidence is
80-90%. For HDCSV or SV alone, the efficacy is at least 70% with 95% confidence. If account is also taken of 57 patients in India who were bitten by rabid animals and subsequently treated with HDCSV and PCECV using the same regimen (Madhusudana et al., 1988), then the proven effectiveness of multisite treatment (as used in the current study) is 80-90%, with 95% confidence.

By July 1988 more than 10,000 patients had been treated at the Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok with a slightly modified multisite ID regimen - currently, 4 x 0.1ml ID injections are given on days 0 and 3 instead of 8 x 0.1ml ID injections on day 0; the day 7, 28, and 91 injections remain unchanged (Wilde & Chutivongse, 1988). The number who were genuinely exposed to rabies is uncertain but at the QSMI rabies is confirmed in approximately 55% of the more than 5,000 animal specimens examined annually following bites to some 19,000 patients. Thus 1170 to 5500 of the 10,000 treatments were probably given following genuine rabies exposure, with only one death which occurred after a 6 day delay between exposure and treatment and an incubation period of 24 days (Wilde & Chutivongse, 1988). This recent Thai data suggest that multisite ID vaccination is at least 98-99% effective in preventing rabies, moreover its efficacy is comparable to the current 5 or 6 x 1.0ml dose regimens.

Cost estimates of the of various postexposure schedules have been provided by Nicholson (1981b) and more recently by Chutivongse & Wilde (1987) who related costs to the minimum daily labourer’s wage in Thailand. There, SMBV costs $9 US per course, and a 5-dose course of HDCSV is $156 US. The newer PVRV and PCECV cost (in Thailand) $55 and $67 US respectively. Multisite ID treatment with these second generation tissue culture vaccines costs approximately $16-20, still twice as expensive as SMBV, but treatment with SMBV requires 13 more clinic visits than the multisite regimen, and the additional cost of the multisite regimen is offset by a loss in earnings (minimum daily labourer’s wage, approximately $2.5-3
US/day) and by occasional neuroparalytic reactions to SMBV.

43. Study 15 - Agreement between the RFFIT and ELISA for rabies antibody

512 sera from patients and volunteers in study 12 and 784 sera from study 14 were used to compare the RFFIT and ELISA. Study 12 included 30 subjects given HRIG, which would be detected in this ELISA, whereas study 14 included patients treated with equine antirabies serum, which would not be detected. In study 12, 8 negative-control sera provided the baseline for measuring antibody by ELISA and the threshold titre for antibody was 1 in 20; the threshold in the RFFIT was 0.5 IU/ml. In study 14, 3 pools of day 0 sera from study 13 were used as negative controls and the threshold titre was 1 in 200. The RFFIT in study 14 was more sensitive than in study 12 and the threshold was 0.1 IU/ml. Regression analysis was undertaken using log_{10} neutralising antibody titres in IU/ml and log_{10} of the reciprocal ELISA titres; values of <1 were taken as 0.2, a titre of <0.5 IU/ml was taken as 0.1, and <0.1 IU/ml was taken as 0.02.

Part (a) of Table 10.39 shows the overall agreement in study 12 between ELISA and RFFIT for sera positive or negative by either method. There was a 90.6% agreement with sera from HDCSV recipients, with 9.2% of the 426 specimens being positive by ELISA and negative by RFFIT. The agreement was less good (77.5%) with sera from SV recipients, of whom 22.5% had detectable IgG antibody in the absence of neutralising antibody. To quantitate the relation between the RFFIT and ELISA more precisely, the line of linear regression missing the origin and the correlation coefficients were determined. There was highly significant correlation between the two tests with sera from both HDCSV (p ≤0.0001, r = 0.79) and SV recipients (p ≤0.0001, r=0.67), but the slope of the regression line was steeper for HDCSV (0.372; standard error, 0.014) than SV sera (0.315; standard error, 0.037) (Figure 10.17) suggesting that SV evokes relatively more non-neutralising IgG antibody than HDCSV.
Table 10.39 Study 15; Agreement between ELISA and virus neutralization (RFFIT) for sera either positive or negative by both methods.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>No. of sera titrated</th>
<th>% (+)ve or (-)ve by either test</th>
<th>% (+)ve by ELISA and (-)ve by RFFIT</th>
<th>% (+)ve by RFFIT and (-)ve by ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Study 12</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HDCSV</td>
<td>426</td>
<td>90.6</td>
<td>9.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Semple</td>
<td>98</td>
<td>77.5</td>
<td>22.5</td>
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<tr>
<td>(b) Study 12, threshold for antibody - 1 in 200</td>
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<tr>
<td>HDCSV</td>
<td>409</td>
<td>88.5</td>
<td>0.2</td>
<td>11.3</td>
</tr>
<tr>
<td>Semple</td>
<td>375</td>
<td>91.5</td>
<td>5.6</td>
<td>2.9</td>
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<tr>
<td>(c) Study 12, threshold for antibody - 1 in 200, or ≥ 5-fold increase in titre compared with day 0.</td>
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<tr>
<td>HDCSV</td>
<td>409</td>
<td>95.6</td>
<td>0.2</td>
<td>4.2</td>
</tr>
<tr>
<td>Semple</td>
<td>98</td>
<td>92.5</td>
<td>5.6</td>
<td>1.9</td>
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a, false-positive day 0 sample by ELISA; b, includes one false-positive day 0 sample by RFFIT and 16 x day 7 sera, of which 13 were from EARS recipients; c, includes 6 x day 7 sera from EARS recipients.

Table 10.39(b) shows that there was approximately 90% overall agreement in study 14 between the results by ELISA and RFFIT when the 'mild' and 'severe' groups were aggregated. On day 7, 29 of 45 specimens that were positive for neutralising antibody and negative for IgG antibody (i.e., did not have a titre ≥ 1 in 200) showed ≥ 5-fold increases in titre compared to the day 0 levels. Table 10.39(c) shows that the overall agreement between the two serological tests rose to approximately 95% when the threshold was taken as either 1 in 200 or a ≥ 5-fold increase in titre compared to the day 0 value. Only 1 of 409 sera (a day 0 sample) from HDCSV patients was false-positive for IgG. 21 (7.0%) of 298 sera collected from SV patients on day 7 or later (representing 5.6% of all 375 SV sera) were positive for IgG but negative for neutralising antibody. Altogether 24 of 784 sera were positive for neutralising antibody and negative for IgG. One was a false-
positive day 0 result by the RFFIT and 20 of 22 day 7 samples were collected after passive immunisation with EARS.

To quantitate the relation between the RFFIT and ELISA in study 14 more precisely, the line of linear regression missing the origin and the correlation coefficients were determined. Analysis again showed a highly significant correlation between the two tests for both the HDCSV ($p \leq 0.0001$, $r = 0.89$) and SV groups ($p \leq 0.0001$, $r = 0.75$), the correlation coefficient was again less for the SV group, and the slope of the regression line was again steeper for the HDCSV group (.554) than SV (.338) (Figure 10.18).

The analyses showed extremely good correlation between the two serological tests. Virtually all of the specimens that were positive for neutralising antibody and negative for IgG were collected on day 7 and the discrepancy probably reflects the presence of both equine antibody and/or IgM neutralising antibody, neither of which would be detected in this ELISA. In both studies the correlation between the two tests was less for sera collected after SV than HDCSV and the slopes of the regression lines were less after SV than HDCSV. Thus in Semple recipients, titres measured by ELISA were associated with lower neutralizing titres than were found after HDCSV. This probably reflects the different nature of the two vaccines. While tissue culture vaccines are prepared from infected cell-supernatants, neural vaccines are derived from cellular debris which is packed with nucleocapsids in addition to intact virions and glycoprotein.
Figure 10.17. Regression plots of log_{10} neutralising antibody and IgG antibody titres. Upper panel, sera from HDCSV recipients; lower panel, sera from SV recipients.
Figure 10.18. Regression plots of log$_{10}$ neutralising antibody and IgG antibody titres. Upper panel, sera from HDCSV recipients; lower panel, sera from SV recipients.
D. CONCLUSIONS
CHAPTER 11.
CONCLUSIONS

1. Introduction

Urban rabies, a major public health problem in the Third World, has been poorly controlled by canine vaccination programmes and the elimination of strays. Accordingly, the vaccination of man with neuro-tissue vaccines remains the principal method of preventing human rabies, but the vaccines are generally of low potency, they cause frequent, serious adverse reactions, and are virtually useless following the most severe exposures. Avian rabies vaccines were also poorly immunogenic and caused frequent allergic reactions. The adaptation to growth of rabies virus to tissue culture heralded the development of a potent human diploid cell culture vaccine. Unfortunately HDCSV is very expensive and although it was shown to be highly effective in postexposure trials in Germany and Iran, the new vaccine and the established treatment regimen offered little immediate comfort to the needy in the Third World. These studies addressed the possibility of developing effective economical regimens for pre- and postexposure prophylaxis suitable for use in the deprived areas of the world.

2. General conclusions

Rabies neutralising antibody has long been invaluable in assessing the humoral response to rabies vaccines and in establishing optimum regimens for pre- and postexposure prophylaxis. The MNT is time-consuming, labour intensive, and expensive and stretched the resources at the Lister Institute. Accordingly other assays were considered. The Lister Institute did not have the equipment necessary for immunofluorescence, so the first strategy was to develop and assess an in vitro neutralisation test based upon the interference phenomenon. The experiments show
that the interference phenomenon can be used for a highly reproducible assay for rabies neutralising antibody. There was good agreement between the interference inhibition test (IIT) and the MNT. Unfortunately the assay had only limited application due to new restrictions on the handling of live rabies virus at the Lister Institute. Nonetheless the results revealed strain-dependant differences in the ability to induce interference and these were explored further in an attempt to better understand the variable behaviour of street rabies virus in man and other animals. The data show that intrinsic interference, not interferon, is the mechanism underlying heterologous interference in GL-V3 monkey kidney cells. There was no evidence that the property of interference was associated with any subsequent inhibition of rabies virus replication or dissemination, or ultimately to the evolution of disease.

The introduction of the more stringent laboratory procedures prevented further work on the IIT and interference phenomenon at the Lister Institute and led to the development of the rabies ELISA. The ELISA was highly reproducible and there was good, but imperfect, agreement between the ELISA and MNT, and ELISA and RFFIT. Several factors were responsible for the discrepancy. First, it occurred with early post-vaccination sera when an IgM response predominates; it also occurred shortly after passive administration with EARS which is undetectable using the anti-human IgG conjugate. Secondly, the agreement between the ELISA and RFFIT was less close for sera collected after NTV than HDCSV - this suggests that the ELISA measures antibodies other than those directed against the glycoprotein and that they are often present at high titre following NTV.

We can reasonably speculate that rabies IgG antibodies are at least as good, if not a better guide of immunity than neutralising antibody, particularly after NTV and shortly after primary immunisation. This supposition is supported by two observations. Dietzschold et al (1987) recently showed that an immune response to the ribonucleoprotein (RNP) is involved in protection - possibly this RNP response
is better correlated with the total IgG responses (i.e., to both the glycoprotein and RNP, which are probably both measured by ELISA) than to virus neutralising antibody, which is directed against the surface glycoprotein. Moreover, mice can be protected against rabies by passively administered IgG neutralising antibody, but not IgM (Turner, 1978, Mifune et al, 1980), and, unlike the ELISA, the RFFIT and MNT do not distinguish between IgG and IgM antibodies.

The ELISA has the benefit of being quick, inexpensive, and straightforward. The technology was readily transferred to the Third World and the results were used to assess vaccine potency, new treatment regimens, and the response to 'routine' immunisation. The need for the latter was recently highlighted at an informal WHO meeting, and a simple semi-quantitative version of the ELISA, suitable for field use, is currently being assessed in Germany.

The clinical studies assessed the clinical, humoral, cell-mediated, and interferon-responses to HDCSV. HDCSV was well tolerated by the IM, SC, and ID routes and with the exception of headache, which occurred in 6% of subjects after IM and SC injections, there was no consistent association between generalised symptoms and vaccination. Local reactions occurred frequently with the ID regimens but were clinically insignificant. One patient in Study 1 and two in Study 14 developed urticarial reactions shortly after vaccination giving an overall allergic reaction rate of 3 per 720 vaccinees, i.e., more than the 11 per 10 000 vaccinees reported in the United States (MMWR, 1984). These allergic reactions are caused by the β-propiolactone-treated human serum albumin component of HDCSV and are less likely features of the new, more purified products available today.

Recommendations for pre-exposure immunization advise that a minimum antibody titre of 0.5 IU/ml should be attained 4 weeks after the last inoculation. Twentyeight days after a second injection by either the IM or ID routes, 100% of vaccinees had titres in excess of 0.5 IU/ml. The proportion of people who were without antibody 6-24 months after immunization were similar whether the IM or
ID routes were used and it is questionable whether the significantly higher titres that developed after IM immunization were clinically important. There was no batch to batch variation in antibody responses, or sex difference, and no clinically significant effect of age. We may therefore conclude that two doses of vaccine given 28 days apart by the IM or ID route are adequate for pre-exposure prophylaxis where the risk of exposure is low. Moreover, there is little or no justification for assaying the antibody responses of low-risk subjects. The results of the MRC studies have since been confirmed by many different workers and the ID route of immunisation for pre-exposure prophylaxis is now officially recommended. It is possible that pre-exposure immunisation with the newer less expensive tissue culture vaccines is cost-effective in areas where the prevalence of rabies and post-exposure treatment are both especially high.

Study 1 showed that a third injection reinforced the humoral response so that antibody was still present in 100% of vaccinees at 2 years and in 86% at three years. However, a few vaccinees had titres of only 0.5 - 1.0 IU/ml three months after the third injection and it is therefore necessary to monitor the antibody titres of individuals at high risk of exposure at 6-monthly intervals and give boosters as required.

A single booster generally evoked a marked and rapid anamnestic response irrespective of the primary regimen or the timing and route of re-immunization or initial antibody titre. Two of 150 (1.3%) persons failed to develop titres in excess of 1.0 IU/ml. In view of this and similar data, the WHO Expert Committee on Rabies has made appropriate recommendations concerning the immunization of exposed persons who have been vaccinated previously.

Study 2 showed that the kinetics and magnitude of the antibody response to 4 × 1.0ml injections were not statistically different to one-tenth the amount administered ID (on days 0, 3, 7, and 14). However, the group-sizes were small and this could readily mask real differences between the regimens under study. Indeed, the day 14 GMTs differed by more than two-fold and Study 12, which also
compared the same regimens, revealed significantly lower IgG and neutralising antibody titres after ID immunization. Of all the regimens evaluated in Study 12, the single-site ID regimen evoked the slowest antibody response and is therefore unacceptable, except possibly during the most severe vaccine shortage. Nonetheless, single site ID vaccination gave antibody titres superior to those after Semple vaccine and this regimen is now used by several workers in East Africa and Thailand.

Studies 3 to 6 concerned the possible role of vaccine-induced interferon in postexposure protection. Interferon induction was inconsistent and since the timely administration of vaccine is generally protective, we can reasonably conclude that interferon induction of is not essential for protection but that it is a useful adjunct to therapy. These conclusions are supported by observations in animals (e.g. Baer et al, 1979; Pille & Matevoysan, 1985). The data further showed that the less purified vaccine induced the higher titres; that there is a marked dose-response effect; that the response to boosters is much less than after primary immunisation; and that the humoral and interferon responses are independent. Most of these observations have since been substantiated in animals or man (Mifune et al, 1980; Bijok et al, 1984; Pille & Matevoysan, 1985; Fornosi et al, 1985).

The association between antibody and protection is well established for pre-exposure prophylaxis, but is less clear for postexposure prophylaxis. If a cell-mediated response is important, then candidate abbreviated regimens must be shown to be at least as effective as the 6 x 1.0 ml dose regimen currently recommended by WHO. Study 7 showed that lymphocytes from 80% of recipients of the first four 1.0 ml doses of the WHO regimen are stimulated in vitro by HDCS antigen. Study 8 further showed that the response is T-cell-mediated and is detectable from days 14-42 in 80% of people after 8 x 0.1 ml ID injections at multiple sites. These data have been confirmed and extended by other investigators (Bijok et al, 1985; Ratavongsiri et al, 1985; Phanuphak et al, 1987). Interestingly, the Thai group has shown that multisite ID vaccination evokes a more rapid blast-transformation
response than the conventional IM regimen which employs larger volumes of vaccine. These and other data (Santos et al, 1980; Ramanna & Pal, 1980; Veiga et al, 1987) indicate that most vaccinees develop cell-mediated immune responses after potent rabies vaccines, suggesting an extremely important role of CMI in postexposure protection. It is difficult to interpret the results of animal studies, since they are obtained using models very remote from the usual circumstances of infection. Nonetheless, experiments in nude athymic mice indicate an essential role of T-cells in postexposure prophylaxis (Turner, 1976; Mifune et al, 1981).

Study 8 also explored the use of an intradermal jet injector. Bleb formation was poor using the injector, suggesting that most of the vaccine went subcutaneously. Moreover, the titres of IgG and neutralising antibody were both lower than was obtained with a needle and syringe. These observations have been confirmed and extended by Bernard et al (1982b). In view of the reduced antibody responses and the possible transfer of HIV and other pathogens, the use of the jet injector is considered wholly inappropriate.

Study 8 confirmed the observation in study 5 that 8 x 0.1ml injections given in separate ID sites on one occasion induce high titres of virus neutralising antibody rapidly. Study 9 confirmed the value of the ELISA and showed that intradermal immunisation could be carried out successfully in a busy immunisation centre. Shortly thereafter Study 10 showed that postexposure, multisite ID, administration of HDCSV completely protected rabbits against intranuchal infection with street rabies virus. Thus we concluded that the success with the ID route could be reliably reproduced by others, that a 'single' multisite ID injection evoked a T-cell mediated response and substantial titres of antibody, and that the multisite ID regimen was at least as effective as the conventional method of immunisation. The scene was then set for the subsequent studies in Pakistan and Thailand.

Study 11 again proved the usefulness of the ELISA and, importantly, showed that lyophilized HDCSV could withstand the rigors of transport and storage at high
ambient temperature in the Third World and retain its immunogenicity by the ID route.

Studies 12 and 13 compared different abbreviated economical regimens for postexposure prophylaxis in volunteers and patients at 'low' risk of exposure. There was a beneficial effect, in terms of early antibody production, of administering 8 x 0.1-ml ID injections as compared to four. There was also a beneficial effect of bringing forward the second injection from day 14 to day 7, and the response to ID vaccination was greater than to the same amount of vaccine administered IM with an aluminium hydroxide adjuvant. HRIG tended to suppress the active antibody response. Overall, these studies showed that multisite ID vaccination was clinically acceptable and that it rapidly evoked high titres of antibody - comparable in height and persistence to IM vaccination, but at a fraction of the cost.

Study 14 evaluated a multisite ID regimen in Thai patients who all had penetrating lesions by rabid animals. 78 were treated with HDCSV and 77 were given Semple vaccine. All survived. The degree of protection afforded is difficult to assess since the risk of developing rabies after a bite by a rabid animal is uncertain. Assuming 15% incidence of rabies in non-vaccinated subjects, we can conclude that the proven efficacy of treatment in this study, with 99% confidence, was 80-90%. This rises to 98-99% if we also take into account patients treated subsequently by other investigators. This estimate accords well with the general experience of HDCSV and suggests that this more economical method of treatment is at least as effective as the 5 or 6 dose WHO regimen.

All of the studies reported were carried out with HDCSV. Less expensive vaccines have since been developed and are marketed on a global basis. Unfortunately the new vaccines are still too expensive ($50-60 US for a 5 or 6 dose course) for those most in need. The combination of the new vaccines and the more economical schedules of immunisation reduces the cost to $16-20 US, but this is still twice as expensive as SMBV. However, it must not be forgotten that the use of NTV necessitates up to 24 injections, which has substantial cost implications, and
that NTVs cause unacceptable reactions. Moreover, NTV is generally acknowledged
to be less effective than the potent tissue culture vaccines, and this also has cost
implications. We can therefore conclude that there is little or no difference in end-
cost between the two therapies and that my goal of developing an effective economical
regimen for use in the Third World has been accomplished. It is rewarding to note
that multisite ID vaccination, like pre-exposure ID vaccination before it, is
increasingly being used. We can hopefully anticipate that the new genetic
engineering techniques will further reduce the cost of treatment and obviate the need
for multiple ID injections.
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Summary

This thesis studies the efficacy of human diploid cell strain rabies vaccine (HDCSV) in man. After a definition of rabies and a historical introduction a searching critical review is presented of all published work on the epidemiology of rabies, the virus, its replication, transmission and the pathogenesis; of human rabies, its pre- and postexposure prophylaxis; the adverse effects of treatment, and an assessment of immunity. The review reveals a desperate need for improved methods of treatment, setting the scene in 1975 when the studies began. Following a description of the volunteers, materials and methods is an account of rabies virus interference and the interference inhibition test which was developed as an alternative to the mouse neutralisation test (MNT). Restrictions in the use of rabies virus led to the rabies enzyme-linked immunosorbent assay (ELISA) described and used in later studies. Studies 1, 2, 9 and 11 show that HDCSV is well tolerated by the intramuscular and intradermal routes and that both reliably induce high antibody titres, irrespective of age, sex, vaccine batch, ambient temperature, and passive immunisation. Studies 3 - 6 show that primary immunisation, not boosters, generally induces circulating interferon and that the interferon and humoral responses are poorly correlated. Studies 7 and 8 reveal a T-cell-mediated blast-transformation response in most vaccinees. Studies 9 and 10 show that satisfactory seroconversion rates are possible in busy centres using the intradermal route and that once only, postexposure, intradermal administration of 4 x 0.2ml HDCSV protects rabbits from street rabies virus. Studies 12 - 14 evaluate abbreviated candidate schedules of immunisation in Thais, one - a multisite intradermal schedule - was given to 78 patients bitten by rabid animals. A further 77 patients were treated with Semple vaccine. All patients survived. In conclusion, HDCSV is considered safe, thermostable and antigenic, and the intradermal route provides effective economical alternatives to the intramuscular route for both pre- and postexposure prophylaxis.