STUDIES ON THE PATHOGENESIS OF LYMPHOCYTIC

CHORIOMENINGITIS VIRUS INFECTION

BY

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A THESIS SUBMITTED FOR

THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN THE AUSTRALIAN

NATIONAL UNIVERSITY

JANUARY 1970
The experiments reported in Section VI were carried out in collaboration with Dr. C. A. Mims. The histological sections were prepared by Mr. R. G. Hill. The remainder of the work described in this thesis was carried out by me.

Department of Microbiology

John Curtin School of Medical Research
## CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II. MATERIALS AND METHODS</td>
<td>6</td>
</tr>
<tr>
<td>III. TESTS FOR HAEMAGGLUTINATION BY LYMPHOCYTIC CHORIOMENINGITIS VIRUS</td>
<td>16</td>
</tr>
<tr>
<td>Suckling mouse brain preparations</td>
<td>18</td>
</tr>
<tr>
<td>Tissue culture preparations</td>
<td>18</td>
</tr>
<tr>
<td>Tests for haemagglutination</td>
<td>19</td>
</tr>
<tr>
<td>IV. THE RESPONSE OF MICE TO THE INTRAVENOUS INJECTION OF LYMHOCHYTIC CHORIOMENINGITIS VIRUS</td>
<td>23</td>
</tr>
<tr>
<td>1. INFECTION OF VISCERAL ORGANS</td>
<td>24</td>
</tr>
<tr>
<td>Intravenous injection of WE₃ strain LCM virus</td>
<td>24</td>
</tr>
<tr>
<td>Immunofluorescence and histological examination of the liver</td>
<td>25</td>
</tr>
<tr>
<td>Immunofluorescence and histological examination of the spleen</td>
<td>31</td>
</tr>
<tr>
<td>Immunofluorescence and histological examination of other organs</td>
<td>34</td>
</tr>
<tr>
<td>Viral replication cycle times in infected macrophages and hepatic cells</td>
<td>36</td>
</tr>
<tr>
<td>Effect of antiserum on the development of necrotic lesions</td>
<td>38</td>
</tr>
<tr>
<td>Intravenous injection of Armstrong strain LCM virus</td>
<td>38</td>
</tr>
<tr>
<td>Clearance of LCM virus from the blood</td>
<td>39</td>
</tr>
<tr>
<td>Adult mice</td>
<td>40</td>
</tr>
<tr>
<td>Infant mice</td>
<td>41</td>
</tr>
</tbody>
</table>
### V. The Response of Mice to the Intravenous Injection of Lymphocytic Choriomeningitis Virus

2. Infection of the Central Nervous System

<table>
<thead>
<tr>
<th>Injection of Adult Mice</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>51</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Injection of Infant Mice</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>52</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Injection of Infant Mice</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>56</td>
</tr>
</tbody>
</table>

### VI. The Pathogenesis of Lesions in the Lymphoid Tissues and Liver of Mice Infected with Lymphocytic Choriomeningitis Virus

<table>
<thead>
<tr>
<th>Spleen Lesions in Mice Infected with LCM Virus</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>72</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Factors Involved in the Production of Spleen Lesions in Mice Infected with LCM Virus</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>76</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lymph Node Lesions in Mice Infected with LCM Virus</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>79</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Thymus Lesions in Mice Infected with LCM Virus</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>81</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Liver Lesions in Mice Infected with LCM Virus</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>84</td>
</tr>
</tbody>
</table>

### VII. The Adoptive Immunization of Mice Immunologically Tolerant to Lymphocytic Choriomeningitis Virus

<table>
<thead>
<tr>
<th>Adoptive Immunization of LCM Virus Carrier Mice</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>93</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Deposition of Immunoglobulin in the Kidneys of LCM Virus Carrier Mice</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>94</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Evidence for the Presence of Circulating Infectious Virus-Antibody Complexes in LCM Virus Carrier Mice</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>99</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Footpad Injection of LCM Virus</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>134</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Intraperitoneal Injection of LCM Virus</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>159</td>
</tr>
</tbody>
</table>
VIII. THE EFFECTS OF BORDETELLA PERTUSSIS VACCINE AND FREUND'S ADJUVANT ON THE RESPONSE OF MICE TO INFECTION WITH LYMPHOCYTIC CHORIOMENINGITIS VIRUS

- Effects of pertussis vaccine on the response of mice to LCM virus infection
  - Intracerebral infection
  - Intraperitoneal infection
  - Footpad infection
- Effect of pertussis vaccine on antibody production
- Effect of pertussis vaccine on the cellular hypersensitivity response
- Effects of Freund's adjuvant on the response of mice to LCM virus infection

IX. THE RESPONSE OF MICE TO THE FOOTPAD INJECTION OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS

- Primary footpad injection of LCM virus
  - Factors involved in the production of primary footpad swelling
- Footpad injection of LCM virus in sensitized mice
  - Factors involved in the production of hypersensitive footpad swelling

X. THE RESPONSE OF MICE TO INFECTION WITH DIFFERENT STRAINS OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS

- Intracerebral injection of LCM virus
- Footpad injection of LCM virus
- Intraperitoneal injection of LCM virus
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intravenous injection of LCM virus</td>
<td>160</td>
</tr>
<tr>
<td>Injection of carrier virus</td>
<td>162</td>
</tr>
<tr>
<td>Response of neonatal mice to infection with different strains of LCM virus</td>
<td>163</td>
</tr>
<tr>
<td>Clearance of different strains of LCM virus from the blood in adult and infant mice</td>
<td>165</td>
</tr>
<tr>
<td>Infection of peritoneal macrophages by different strains of LCM virus</td>
<td>167</td>
</tr>
<tr>
<td>XI. THE RESPONSE OF MICE OF DIFFERENT STRAINS TO INFECTION WITH LYMPHOCYTIC CHORIOMENINGITIS VIRUS</td>
<td>179</td>
</tr>
<tr>
<td>Response to intracerebral infection with LCM virus</td>
<td>180</td>
</tr>
<tr>
<td>Response to intraperitoneal infection with LCM virus</td>
<td>184</td>
</tr>
<tr>
<td>Response to the footpad injection of LCM virus</td>
<td>186</td>
</tr>
<tr>
<td>XII. FINAL DISCUSSION</td>
<td>200</td>
</tr>
<tr>
<td>XIII. SUMMARY</td>
<td>210</td>
</tr>
<tr>
<td>XIV. REFERENCES</td>
<td>214</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I am greatly indebted to my supervisor, Dr. Cedric Mims, for his guidance and encouragement. His advice and constructive criticism were freely given and were of invaluable assistance.

My thanks are due to my colleagues in the Department for many stimulating discussions.

The skilled histological assistance of Mr. R. G. Hill is gratefully acknowledged.

This work was carried out during the tenure of an Australian National University Research Scholarship.
TO

Anne, Andrew

and

Ellena

The world is still full of mystery, and it will be long before we understand much, even when we limit ourselves to the mere mechanism of life and do not approach that great mystery of all: that we, as conscious beings, are capable of pondering it all....

Dr. Vannevar Bush
The world is still full of mystery, and it will be long before we understand much, even when we limit ourselves to the mere mechanism of life and do not approach that greatest mystery of all: that we, as conscious beings, are capable of pondering it all.

Dr. Vannevar Bush
I. INTRODUCTION

Although the practical control of infectious diseases has frequently depended on advances in public health measures, immunization and chemotherapy, an understanding of the pathogenesis of infectious diseases remains of fundamental importance. In lymphocytic choriomeningitis virus infection in its natural host, the variable host-parasite relationships which may occur provide a remarkable opportunity for a many-faceted study of the pathogenesis of the disease.

Lymphocytic choriomeningitis (LCM) virus was so named by its discoverers (Armstrong & Lillie, 1934) because it caused a meningitic illness, with a mononuclear cell infiltration in the meninges and choroid plexuses. Its natural host is the mouse, but it may infect many other mammals, including man. Although meningitis is commonly used as an index of infection in experimental animals, LCM virus may grow in most tissues of an infected animal and death can occur without obvious signs of central nervous system infection. In vitro studies have shown that LCM virus is basically non-cytopathic for mouse cells (Hotchin & Cinits, 1958; Benda & Činátl, 1962). The work of Rowe (1954) suggested
that the essential factor in the production of disease in LCM virus infection was the cellular immune response, and this concept was more firmly established by Hotchin (1962a). In animals infected with LCM virus, sickness and death are not caused by direct cell destruction by the virus, but appear to result from an interaction between sensitized lymphoid cells and infected tissue cells. When the cellular immune response is depressed by X-irradiation, neonatal thymectomy or treatment with antimetabolites, cortisone or antilymphocyte serum, there is a marked reduction in severity of the disease caused by infection with LCM virus.

Thus, LCM virus infection exemplifies a situation in which there may be a virtually harmless host-parasite relationship, until the onset of an immune response to the infectious agent results in pathological changes in the host. However, little is known about the histopathogenesis of LCM virus infection, which is of basic importance in an understanding of the disease process. Most investigations of the development of this infection have been carried out by routine histological techniques or organ titration methods (Lillie & Armstrong, 1945; Rowe, 1954; Lehmann-Grube, 1964a) which give no information about the site of initiation of infection, the spread of infection or the types of cell supporting...
virus growth. In contrast, the immunofluorescence technique enables study of the distribution of virus at the cellular level and analysis of the process of infection in a particular tissue. A limited investigation using immunofluorescence in mice acutely infected with LCM virus was made by Wilsnack and Rowe (1964), but the pathogenesis of the infection was not studied.

In the present work, the immunofluorescence technique has been used in conjunction with histological examination and virus titration methods, in order to further elucidate the histopathogenesis of LCM virus infection in the mouse. Studies have been made of the passage of virus from the blood into the central nervous system and the viscera, in particular the liver and spleen, and the development and course of the infection. Mice infected with LCM virus were found to develop severe necrotic lesions in the lymphoid tissues and in the liver. In view of the probable importance of immunological mechanisms in the production of these lesions, their nature and pathogenesis have been investigated.

A further section describes attempts which have been made to develop a haemagglutination test for LCM virus, to simplify the determination of virus titres and to enable the detection of antibody by haemagglutination-inhibition.
Another important aspect of LCM virus infection in mice is the development of immunological tolerance to the virus following intrauterine or neonatal infection (Traub, 1960b; Lehmann-Grube, 1964b). The mice remain healthy for long periods, despite the persistence of infectious virus throughout the body. Reversal of the immunological tolerance of these LCM virus carrier mice, with restoration of the immune response, could be expected to result in disease and perhaps death. Experiments have been carried out to determine the effects of adoptive immunization of LCM virus carrier mice. The work of Volkert (1965) has been extended, using immunofluorescence and histological examination.

In subsequent sections, different aspects of the pathogenesis of LCM virus infection have been studied, in order to further clarify the pathogenetic mechanisms involved. Because of the marked effects of adjuvants on the cellular immune response (White, 1967), the effects of Freund's adjuvant and Bordetella pertussis vaccine on LCM virus infection have been investigated. The primary footpad swelling which occurs in mice after footpad infection with LCM virus (Hotchin, 1962b) has been studied in order to determine the mechanisms of its production. A hypersensitive footpad swelling has been described in
mice previously infected with LCM virus, and its relation to the degree of sensitization to LCM virus has been investigated. Another section is devoted to studies of differences in the infectious process, and in the host response to infection, when different strains of LCM virus are used. Finally, the response of different strains of mice to infection with LCM virus has been investigated, in relation to the factors involved in the pathogenesis of the disease.

Microbial fauna of the colony was not carried out, but it was found to be free of mouse tapeworm, Sparganum coccoides. LCM virus and ectromelia virus, Suckling mouse diarrhea and threadworm infestation were sometimes present, however, and it can be described as a "low pathogen" colony. Unless otherwise specified, Wistar mice were used in the experiments reported.

Inbred mice of the C57BL, C3H and CBA strains (Staats, 1964) and also Bagg inbred mice, were from similar low-pathogen colonies. Bagg mice were originally obtained from the Roswell Park Memorial Institute, Buffalo, New York (Briody et al., 1958), and strain A mice were obtained from Castle Hill Animal House, University of Sydney.

Virus

The W3 and Armstrong strains of LCM virus
II. MATERIALS AND METHODS

Mice

Outbred mice were of the Walter & Eliza Hall Institute (WEHI) multi-coloured strain. The colony was derived by caesarean section and rat foster-mother nursing, and had been maintained in the University Animal Breeding Establishment since 1952. The mice were fed with commercial high protein mouse cubes. A definitive study of the microbial fauna of the colony was not carried out, but it was found to be free of mouse tapeworm, Eperythrozoon coccoides, LCM virus and ectromelia virus. Suckling mouse diarrhoea and threadworm infestation were sometimes present, however, and it can be described as a "low pathogen" colony. Unless otherwise specified, WEHI mice were used in the experiments reported.

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Virus

The WE3 and Armstrong strains of LCM virus.
(Lehmann-Grube, 1964a) were used, having since undergone several additional passages in guinea pig lung and mouse brain, respectively. The WE3 strain stock virus was the supernate of a lung suspension obtained from guinea pigs six to seven days after subcutaneous injection of virus. Its titre was \(10^8.9\) mouse intracerebral LD50 per ml. The Armstrong strain of LCM virus is a mouse-adapted strain, in that it has been passaged repeatedly in mouse brain. Armstrong strain stock virus was the supernate of a brain suspension obtained from mice five days after intracerebral inoculation, and its titre was \(10^6.7\) LD50 per ml.

"Carrier virus" was also used and is that obtained from the blood or spleen of mice of an LCM virus carrier colony (see below).

**Neonatal Infection**

Less than twenty-four hours after birth, mice were injected intraperitoneally with \(>10^5\) LD50 of WE3 strain LCM virus.

**Carrier Colonies**

C57BL and WEHI LCM virus carrier colonies were initiated in 1966 by injection of neonatal mice with a large dose of WE3 strain LCM virus. Both colonies had passed through several generations by the time mice were used in these experiments.
Intravenous Injection

Adult mice were injected via a tail vein with an inoculum of 0.5 ml. In infant mice, the inoculum of 0.1 ml., coloured with 0.05% trypan blue, was injected into the superior vena cava above the sternal end of the clavicle. Firm pressure for a few seconds after removal of the needle prevented bleeding. Intravenous injection was indicated by rapid development of the blue colour in peripheral blood vessels and in the liver and skin.

Injection into the Common Bile Duct

Mice were anaesthetized by the intraperitoneal injection of Nembutal 2.5 mg. The common bile duct was visualized through a transverse upper abdominal incision with the aid of a dissecting microscope. The inoculum of 0.5 ml., coloured with 0.05% trypan blue, was injected into the duct in a retrograde direction. The incision was sutured with black silk.

Immunization

A small dose of LCM virus was injected intravenously or subcutaneously into the footpad, followed by a larger dose intraperitoneally after 14 days.

Irradiation

Mice received 550 rads of whole-body gamma irradiation from the Cobalt-60 source (Division of Plant Industry, C.S.I.R.O., Canberra) described by Brock (1962).
Adoptive Immunization

Immunized mice were killed three to four weeks after the last inoculation of LCM virus and spleens and lymph nodes were removed into Puck's saline at 0°C. These tissues were chopped with scissors and passed through stainless steel gauze. The cell suspension thus obtained was pooled with peritoneal washings from the mice and the cells were deposited by centrifugation at 135 g for 5 minutes. After resuspension in Puck's saline with added antibiotics, the cells were counted in a haemocytometer and the proportion of viable cells estimated by trypan blue exclusion. An inoculum containing $100 \times 10^6$ viable immune lymphoid cells, together with approximately $10^6$ peritoneal cells, was injected intraperitoneally in each carrier mouse or in normal mice as controls. These cell suspensions were free of detectable virus, as determined by intracerebral inoculation in normal WEHI mice.

In some experiments, femoral bone marrow was obtained from immune mice and approximately $10^6$ cells added to the inoculum of spleen, lymph node and peritoneal cells used for adoptive immunizations. In other experiments, cells for transfer were obtained from acutely infected mice, six days or ten days after an
intracerebral injection of LCM virus.

**Fluorescence Microscopy**

Tissues were frozen in containers in liquid nitrogen and sections six microns thick were cut at -20°C in a Model CTD Harris cryostat (International Equipment Co., Needham Heights, Massachusetts). Thoroughly dried sections were fixed in acetone for 10 minutes at room temperature before staining.

Antiserum to LCM virus was produced in guinea pigs by a single injection of Armstrong strain virus followed by multiple injections of WE₃ strain virus. Crude gamma globulins were obtained by cold methanol precipitation and conjugated with fluorescein isothiocyanate (Sylvana Chemical Co., Orange, New Jersey), 1.25 mg per ml of serum. The conjugate was dialysed against phosphate-buffered saline and non-specific staining was reduced by adsorbing twice with a mixture of rabbit liver and chick embryo fibroblast powders. Fluorescein-conjugated goat antiserum globulin against mouse serum IgG globulin (Microbiological Associates, Bethesda, Maryland) was used to demonstrate mouse antibody. Rhodamine-conjugated bovine plasma albumin (Microbiological Associates) was added to each fluorescein conjugate as a counterstain.
The fixed frozen sections were stained with the appropriate conjugate for 20 minutes, rinsed in phosphate-buffered saline for a similar period and mounted in neutral glycerol. Sections were examined with a Zeiss microscope equipped for fluorescence observations and illuminated with an Osram HBO 200 high pressure mercury vapour lamp. Three sections were examined from each tissue specimen.

As controls, sections of normal tissues were stained in the same way as infected tissues. In all spleens examined, non-specific fluorescence, which appeared yellowish-green and homogeneous, was seen in eosinophils scattered throughout the red pulp and in tingible body macrophages in the white pulp, mainly in the central areas of splenic follicles. This was distinct from the granular, apple-green, specific fluorescence of LCM antigen. Even in unstained sections, variable amounts of non-specific, orange, auto-fluorescent material could be seen in macrophages in the spleen, in Kupffer cells in the liver and in glial cells in the brain.

**Histology**

Tissues were fixed in 10% formol saline, embedded in paraffin, sectioned and stained with haematoxylin and eosin. In some experiments, sections were also stained
with methyl green-pyronin (Unna-Pappenheim), periodic acid-Schiff stain for polysaccharides, Mallory's phosphotungstic acid haematoxylin, Van Gieson-haematoxylin and Mallory's trichrome stain for collagen and connective tissue, and Gomori's silver impregnation for reticulin. Stains for amyloid were 1% aqueous thioflavine T, congo red and methyl violet. Fettrot 7B was used to stain fat in frozen sections of formalin-fixed liver.

**Infectivity Titrations**

Blood was collected in heparinized gelatin saline. Tissues were ground in a frozen mortar, suspended in gelatin saline and centrifuged at 2,500 g for 30 minutes at 0°C, to remove debris. Tenfold dilutions of the supernate were inoculated in 0.03 ml amounts intracerebrally into groups of five WEHI mice. The LD$_{50}$ titres were calculated by the method of Reed & Muench (1938). Some titrations were performed in tissue culture, using the plaque assay method of Wainwright & Mims (1967) with chick embryo fibroblast monolayers. Virus titres are expressed as log$_{10}$ LD$_{50}$ or log$_{10}$ p.f.u. per ml of fluid or per gram of tissue.

**Complement Fixing Antibody Titrations**

The Takátsy microtechnique (Sever, 1962) was used. The sera were inactivated at 56°C for 30
minutes. Serial twofold dilutions, using 0.025 ml amounts, were made in Ca Mg saline in perspex trays. Antigen was prepared by sucrose-acetone extraction of infected suckling mouse brain (Grešíková and Casals, 1963). To each cup were added 3 MHD of antigen and 5 MHD of guinea pig complement and the trays were incubated overnight at 4°C. Sensitized sheep red blood cells were then added, to give a final concentration of 0.5%, and the trays incubated with shaking at 37°C for 90 minutes. They were then held at 4°C for 2 hours and the endpoints taken as 50 per cent haemolysis.

Neutralizing Antibody Titrations

Undiluted, uninactivated serum was incubated with a large amount of virus \(10^7 \text{LD}_{50}\) and complement, for 1 hour at 37°C. The reaction mixture was centrifuged at 2,500 g for 20 minutes, to deposit any virus-antibody complexes formed. The supernate was then titrated intracerebrally in mice and the neutralization index calculated from the reduction in virus titre by the test serum, compared with the control sera. This method overcomes the problem of the possible presence of virus or infectious virus-antibody complexes in the test serum, because their titre would become insignificant in the face of the large amount of added virus.
Footpad Measurements

The footpads of mice were measured by the technique of Ackerman (1964) with dial gauge calipers (Schnelltaster, H.C. Kröplin, Schlüchtern, Hessen, Germany) calibrated to 0.05 mm. The percentage increase in thickness of the infected footpad was calculated in comparison with the normal foot.

Peritoneal Macrophages

Peritoneal washings were obtained from unstimulated mice as described by Mims (1964b), using Eagle's medium containing 20% heat-inactivated calf serum. Heparin was not added to the medium, since it may alter the character of peritoneal macrophages (Mims, 1969a) and clotting was avoided by dilution of the washings. The cell suspensions were dispensed into glass rings sealed to microscope slides with petroleum jelly (Cairns, 1960), and placed in a CO$_2$-gassed, humidified incubator at 37°C. After incubation for several hours, when the macrophages had adhered to the glass, cells not sticking to glass were removed by washing with normal saline. The macrophages were then infected and medium added. After incubation for the required period, the medium was removed and the cell sheet was dried, fixed in acetone and stained with fluorescein-conjugated antibody. At least two replicates were used in each test.
Antilymphocyte Serum

This was produced by the method of Levey & Medawar (1966). Rabbits were injected intravenously with at least $4 \times 10^8$ thymus cells taken from 4-6 week old C$_{57}$BL mice. The injection was repeated two weeks later, and serum taken after another week. Sera were treated at 56°C for 30 minutes before use. Mice were given 2 or 3 daily subcutaneous injections of 0.3 ml, starting on the day of infection with LCM virus. This prevented footpad swelling after footpad infection and pleural and peritoneal fluid production after intraperitoneal infection. It prevented death after intracerebral infection, but mice generally showed some sickness 7 to 12 days after infection.
III. TESTS FOR HAEMAGGLUTINATION BY LYMPHOCYTIC CHORIOMENINGITIS VIRUS

Electron microscopy studies have shown that LCM virus particles are formed by budding from the outer cell membrane and have projections on their surface (Dalton et al., 1968; Compans, R.W., unpublished data). These morphological observations, along with cytochemical and other data (Pfau et al., 1965; Dalton et al., 1968) suggest that LCM virus belongs to a presently unclassified group of lipoprotein-enveloped RNA-containing viruses. Because of the many similarities between LCM virus and several other enveloped RNA-containing viruses which are known to cause haemagglutination, and because LCM virus adsorbs to the red blood cells of infected guinea pigs and mice (Shwartzman, 1944, 1946), the possibility of developing a haemagglutination test for LCM virus was considered. Such a test would be extremely useful because of its simplicity and its adaptability to studies of haemagglutination-inhibition by antibody to LCM virus.

LCM virus shows certain similarities to the arboviruses, which are also enveloped RNA-containing viruses and are of similar size, at least to the smaller LCM virus particles. Filtration and centrifugation experiments (Scott & Elford, 1939; Pfau, 1965) indicated
an LCM virus particle size of 40-60 nm, but the recent electron microscopy observations showed a pleomorphism from 50-200 nm. There have been reports of the transmission of LCM virus by arthropods (Coggeshall, 1939; Shaughnessy & Milzer, 1939; Milzer, 1942; Reiss-Gutfreund et al., 1962). In the first instance, therefore, techniques for the preparation and testing of arbovirus haemagglutinins (Clarke & Casals, 1958; Mussgay et al., 1967) were applied to LCM virus. Subsequently, modifications were made to the techniques according to methods used for haemagglutination with rubella virus (Stewart et al., 1967; Halonen et al., 1967; Holmes & Warburton, 1967) and rabies virus (Halonen et al., 1968), since these are also enveloped RNA-containing viruses which mature at the cell surface. Rubella virus has many characteristics in common with arboviruses (Holmes & Warburton, 1967), as well as some with LCM virus. Both rubella virus and LCM virus cross the placenta to infect the foetus and the infections persist after birth (Mims, 1966a, 1968b; Rawls, 1968). In tissue culture, both viruses exert a similar type of interference to infection with paramyxoviruses (Marcus & Carver, 1965; Wainwright & Mims, 1967). The plaque
<table>
<thead>
<tr>
<th>Preparation of Haemagglutinating Antigen</th>
<th>METHODS USED FOR ARBOVIRUSES</th>
<th>METHODS USED FOR RUBELLA VIRUS</th>
<th>METHODS USED FOR RABIES VIRUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid</td>
<td>Sucrose-acetone extraction of infected suckling mouse brain</td>
<td>BHK-21 tissue culture</td>
<td>BHK-21 tissue culture</td>
</tr>
<tr>
<td>Liquid</td>
<td>Tween 80-ether extraction of infected suckling mouse brain</td>
<td>Kaolin treatment of serum in tissue culture medium</td>
<td>Late harvest of tissue culture fluid</td>
</tr>
<tr>
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<td>pH range with phosphate buffer system</td>
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</tr>
<tr>
<td>Goose</td>
<td>Goose Pigeon Sheep</td>
<td>Goose</td>
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<tr>
<td>37°C</td>
<td>0°C and 4°C</td>
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* Mouse and Guinea pig red blood cells were also used

Table 1. METHODS APPLIED TO TESTS FOR LCM VIRUS HAEMAGGLUTINATION
assay system of Sedwick & Wiktor (1967) was found to be effective for LCM and rubella viruses as well as for rabies virus.

**EXPERIMENTAL**

The methods used in the attempted preparation and testing of LCM virus haemagglutinin were those which have been used successfully for arboviruses, rubella virus and rabies virus (Table 1).

**Suckling Mouse Brain Preparations**

4-day-old WEHI mice were inoculated intracerebrally with $10^6 LD_{50}$ of the WE$_3$ strain of LCM virus and the brains were removed at seven days. Using techniques developed for the preparation of arbovirus haemagglutinins, half were treated by sucrose-acetone extraction (Clarke & Casals, 1958) and half by Tween 80-ether extraction (Mussgay et al., 1967).

**Tissue Culture Preparations**

The BHK-21 baby hamster kidney cell line was used, since these cells have been used to obtain haemagglutinins of rubella virus (Stewart et al., 1967; Halonen et al., 1967; Holmes & Warburton, 1967) and rabies virus (Halonen et al., 1968). The cells were grown as monolayer cultures in petri dishes at 37°C in modified Eagle's medium (Macpherson & Stoker, 1962).
containing 10% unheated foetal calf serum. Maintenance medium contained 2% heat-inactivated foetal calf serum. In cell monolayers inoculated with the WE3 strain of LCM virus, a peak virus titre of $10^6 \text{LD}_{50}$ per ml of tissue culture fluid was reached at three days after infection. The virus was passaged repeatedly in an attempt to obtain increased titres and at the sixth passage, the level of virus in the tissue culture fluid had risen to $10^8 \text{LD}_{50}$ per ml.

For the attempted preparation of haemagglutinin, cell monolayers were washed twice with physiological saline and inoculated with $10^8 \text{LD}_{50}$ of tissue culture passaged virus. Maintenance medium contained 2% heat-inactivated foetal calf serum which had been treated with kaolin (Clarke & Casals, 1958), as used for rubella virus (Stewart et al., 1967; Holmes & Warburton, 1967), to adsorb nonspecific inhibitors of haemagglutination. The tissue culture fluid was removed at three days, centrifuged at 1,000 g for 10 minutes to remove cell debris and tested for the presence of haemagglutinin.

**Tests for Haemagglutination**

The Takátsy microtechnique (Sever, 1962) and the phosphate buffer system used for arboviruses (Clarke & Casals, 1958; Work, 1964), rubella virus (Halonen et
al., 1967) and rabies virus (Halonen et al., 1968) were employed. Blood was collected in Alsever's solution and washed 3 times in dextrose gelatin veronal (DGV) solution. The tests were performed with goose red blood cells, as used for arboviruses (Clarke & Casals, 1958), rubella virus (Stewart et al., 1967) and rabies virus (Halonen et al., 1968). Pigeon and sheep red blood cells were also used, since they have been shown to give haemagglutination with rubella virus (Stewart et al., 1967; Holmes & Warburton, 1967). In addition, the tests were performed with guinea pig and mouse red blood cells, to which LCM virus has been shown to adsorb (Shwartzman, 1944; 1946).

Serial twofold dilutions of the mouse brain or tissue culture preparations were made in perspex trays, in 0.05 ml volumes of borate saline pH 9.0 containing 0.4% bovine albumin. Addition of equal volumes of 0.25% red blood cell suspensions in appropriately-buffered phosphate adjusting diluents enabled the test to be performed at a pH range from 5.75 to 7.4. After shaking, the trays were incubated at 37°C for 1-2 hours, as for arboviruses (Clarke & Casals, 1958).

Results

All tests were negative. That is, no LCM virus haemagglutinin was demonstrable, using methods that are
successful with arboviruses, rubella virus and rabies virus.

Variations in method were therefore tried. The tests were repeated with incubation at 4°C as used for rubella virus (Stewart et al., 1967; Holmes & Warburton, 1967) and at 0°C on an ice slurry, as for rubella virus (Halonen et al., 1967) and rabies virus (Halonen et al., 1968), but they remained negative. A different buffer system, which was found to give optimal haemagglutination with rubella virus (Holmes & Warburton, 1967), was then employed. Goose, pigeon and mouse erythrocytes were suspended in DGV diluent with the pH adjusted by Gomori's tris-maleate buffer, such that when added to dilutions of the test material in DGV diluent, the final pH was 6.2. The results were again negative.

In the case of rabies virus, peak haemagglutinating activity has been found to occur at a later stage than peak infectivity (Halonen et al., 1968). The tissue cultures infected with LCM virus were therefore maintained for six days instead of three days, changing the medium every 48 hours. Haemagglutinating activity was still not detected in the tissue culture fluid.

It was thought possible that LCM virus may have adsorbed to red blood cells in the test system, without
causing detectable haemagglutination. The following test was therefore carried out to investigate whether antiserum to LCM virus would cause haemagglutination by binding to virus adsorbed to red blood cells. Dilutions of tissue culture fluid in DGV buffer at pH7.2 were incubated with guinea pig red blood cells for 90 minutes at 37°C, room temperature and 0°C, and guinea pig antiserum to LCM virus was then added, at a final dilution of 1 in 10. After re-incubation for 1 hour, however, haemagglutination was still not detectable.

Thus, the attempted demonstration of an LCM virus haemagglutinin in mouse brain and tissue culture preparations has so far failed. Tests were carried out with a variety of red blood cells, under a wide range of pH, using methods which have proved successful with arboviruses, rubella virus and rabies virus.
IV. THE RESPONSE OF MICE TO THE INTRAVENOUS INJECTION OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS

1. INFECTION OF VISCERAL ORGANS

Adult mice which are immunologically tolerant to LCM virus, by virtue of intrauterine or neonatal infection, appear healthy despite continually high virus titres in their blood and tissues (Traub, 1939; 1960b; Haas, 1941). In contrast, LCM virus infection of normal adult mice can cause death within seven days and disease and death appear to be due to an immunological conflict within the host (Rowe, 1954; Hotchin, 1962a). There have been a number of investigations of the pathogenesis of LCM virus infection, using routine histological examination (Lillie & Armstrong, 1945; Shwartzman, 1946) and organ assay methods (Rowe, 1954; Lehmann-Grube, 1964a). The only investigation using the immunofluorescence technique in acutely infected mice has been that of Wilsnack & Rowe, (1964). They determined the day of appearance of viral antigen in the brain and meninges after intracerebral infection and in visceral organs after intraperitoneal infection and described the fully developed infection in these tissues. However, no study was made of the pathogenesis of the infection. Because of the
unparalleled advantages provided by the immunofluorescence technique in studying the pathogenesis of infection, it has been used to follow the development and course of infection in the liver, spleen and other tissues of mice injected intravenously with LCM virus. The localization of infected cells has been correlated with the localization of immunoglobulin and the development of histological changes in the tissues.

RESULTS

A. Intravenous Injection of WE₃ Strain LCM Virus.

Adult WEHI mice were injected intravenously with 10⁷LD₅₀ of WE₃ strain virus. The mice remained well until the fifth day, when they developed a hunched posture, ruffled fur, trembling and conjunctivitis, with diminished activity and laboured respiration. The liver, at this stage, was enlarged, pale and faintly mottled. The spleen was congested and enlarged to two or three times the normal size, the pancreas was oedematous and the lungs were congested. Mild pleural and peritoneal effusions were present. By the sixth day, some deaths had occurred. The sick mice exhibited the same clinical and pathological signs as at five days, but to an even more severe degree. However, tail spinning did not cause convulsions with extended hind limbs, as occurs after intracerebral injection of LCM
virus, and dead mice were in a hunched position. All mice infected with this dose of virus died by the eighth day.

At intervals after infection, groups of four mice were killed and the tissues removed for immunofluorescence and histological examination. Until four days after infection, there was little variability between the organs of different mice taken at the same time interval. As a routine, however, the percentages of infected cells in the liver were calculated from the organ showing the most advanced changes at a particular time. A quantitative estimation of infected cells in the spleen was not possible.

**Immunofluorescence and Histological Examination of the Liver**

**Five Minutes.** Small amounts of viral antigen were seen in the cytoplasm of approximately 1% of Kupffer cells, randomly distributed in the liver.

**One Hour.** Viral antigen had disappeared from the liver.

**Nine Hours.** No infected cells could be seen.

**Eleven Hours.** New viral antigen was first detected. Infection was seen in approximately 1% of Kupffer cells.
Figure 1. Liver at twenty-four hours after intravenous injection of $10^7 LD_{50}$ of WE$_3$ strain LCM virus. Infected hepatic cell and infected Kupffer cells. Fluorescent antibody stain. X 600.
Eighteen Hours. There was no change in Kupffer cell infection, but infected endothelial cells were now present in some hepatic and portal veins. No infected hepatic cells could be seen.

Twenty-four Hours. 2-3% of Kupffer cells were infected. Infected single hepatic cells were now detected, scattered randomly through the liver and comprising 0.5 - 1% of hepatic cells (Figure 1). Fluorescence was mainly cytoplasmic, but some nuclear fluorescence was also present.

Thirty-two Hours. Infection had extended to involve about 5% of Kupffer cells and 2% of hepatic cells. The infected hepatic cells were still scattered singly throughout the liver.

Forty Hours. Occasional groups of 2-3 infected hepatic cells were now present.

Forty-eight Hours. 15% of Kupffer cells were infected and still about 2% of hepatic cells. The number of groups of 2-3 infected hepatic cells was approximately equal to the number of single infected hepatic cells seen at twenty-four hours.

Three Days. 30% of Kupffer cells and 5% of hepatic cells were now infected. The infected hepatic cells were still mostly single, with occasional groups of 4-6 cells.
Figure 3. Liver at four days after intravenous injection of $10^7LD_{50}$ of WE$_3$ strain LCM virus. Focus of parenchymal necrosis, associated with infiltrating mononuclear cells. Haematoxylin and eosin. X 400.
Four Days. 50% of Kupffer cells were infected and the proportion of infected hepatic cells had increased markedly to 60% (Figure 2). The foci of infected hepatic cells had increased in both number and size and had interconnected to become semiconfluent. However, some single infected hepatic cells were still observed.

For the first time, abnormal changes were detected in haematoxylin and eosin stained sections (see also Section VI). Small numbers of necrotic hepatic cells were seen, scattered randomly throughout the liver. These appeared singly or in small groups and were usually surrounded by mononuclear cells (Figure 3). The cytoplasm of the degenerating hepatic cells was more homogeneous and more eosinophilic than normal, and the nuclei were undergoing karyolysis, pyknosis or karyorrhexis. Later, fragmentation of the cytoplasm occurred. Around the necrotic foci was often seen a layer of morphologically intact hepatic cells which stained in a more eosinophilic and more homogeneous fashion than normal; these cells were possibly in the early stages of degeneration. The cytoplasm of intact hepatic cells had developed a fine vacuolation. There was a moderate increase in mononuclear cells in the sinusoids and portal tracts. Numerous small foci of these cells were scattered in the sinusoids
Figure 4. Liver at four days after intravenous injection of $10^7$ LD$_{50}$ of WE$_3$ strain LCM virus. Focus of infiltrating cells in sinusoid. Haematoxylin and eosin. X 400.
and a few were seen adhering to the endothelium of veins. Most were large pale mononuclear cells (Figure 4), but small lymphocytes, plasma cells and polymorphonuclear leukocytes were also present. Some of these cells were infected, small numbers were pyroninophilic, and occasional degenerating mononuclear cells were seen in the sinusoids.

Five Days. The proportions of infected Kupffer cells and hepatic cells were 40% and 50% respectively. That is, little change had occurred between four and five days; if anything, the numbers of infected cells had decreased. The necrotic foci were more numerous and larger in size. The necrotic hepatic cells contained only small amounts of viral antigen and lay within foci of intact hepatic cells containing larger amounts of antigen. There was a marked increase in mononuclear cells infiltrating the portal tracts and sinusoids and adhering to venous endothelium, and many of these cells were pyroninophilic. The infiltration was especially marked around the necrotic foci. Thus, foci of necrosis appeared to develop in areas of infected hepatic cells, in association with a mononuclear cell infiltration in the sinusoids.

Sections stained with fluorescent antiserum
Figure 5. Liver at five days after intravenous injection of $10^7 LD_{50}$ of WE$_3$ strain LCM virus. Immunoglobulin in and around focus of parenchymal necrosis. Fluorescent antibody stain. X 200.
against mouse immunoglobulin were compared with similarly stained sections of normal livers. In the normal livers, immunoglobulin was present in the cytoplasm of a small number of mononuclear cells in the connective tissue of the portal tracts. In the infected livers, immunoglobulin, presumably antibody to LCM virus, was seen in foci of parenchymal necrosis (Figure 5) and was most marked around their periphery, often within infiltrating mononuclear cells. Immunoglobulin was also present in some apparently intact hepatic cells, either singly or in small groups, usually adjacent to necrotic foci. The immunoglobulin in these cells was often concentrated around the outer part of the cytoplasm. A moderate number of cells in the portal tracts and scattered cells in the sinusoids also contained immunoglobulin.

The specificity of staining for mouse immunoglobulin was established as follows. Pretreatment with the 7S fraction of goat antiserum against mouse serum IgG globulin (Cappel Laboratories, Downingtown, Pasadena) resulted in marked quenching of fluorescent staining by the anti-mouse conjugate, whereas pretreatment with the 7S fraction of goat antiserum against guinea pig serum IgG globulin (Cappel Laboratories) did not.

Six Days. There were no significant changes in
Figure 6. Liver at six days after intravenous injection of $10^7LD_{50}$ of WE$_3$ strain LCM virus. Advanced parenchymal necrosis. Mononuclear cells adhering to venous endothelium. Haematoxylin and eosin. X 100.
infection, but the necrotic lesions were more advanced and the immunoglobulin staining had increased. The necrotic foci were larger and consisted of areas of necrotic hepatic cells with infiltrating mononuclear cells, some of which showed karyorrhectic changes (Figure 6). Occasional pyroninophilic cells were seen in the necrotic areas and some foci contained a number of erythrocytes. It was now seen that the necrotic lesions tended to be mid-zonal in distribution, and in general, the hepatic cells around portal tracts and central veins were least affected. Gross disturbance of the liver architecture had occurred. Intact hepatic cells were vacuolated and many sinusoids and central veins had collapsed. There was a marked increase in the mononuclear cell infiltration in the portal tracts and sinusoids and many more cells, mostly large mononuclears, were now adhering to venous endothelium (Figure 6); many of these cells were pyroninophilic. Large amounts of cellular debris, in the form of necrotic cells and cytoplasmic and nuclear fragments, were present in the veins of the liver, especially the hepatic veins.

In summary, following the intravenous injection of LCM virus, there was virus growth in Kupffer cells by eleven hours. Infected hepatic cells were not detected until twenty-four hours, and the number of infected
Figure 7. Spleen at twenty-four hours after intravenous injection of $10^7 LD_{50}$ of WE$_3$ strain LCM virus. Infected cells in red pulp and in perifollicular region. Fluorescent antibody stain. X 125.
hepatic cells increased much more slowly than the number of infected Kupffer cells. Necrosis of infected hepatic cells began at four days and was associated with an infiltration of mononuclear cells. Mouse immunoglobulin was present in some of the infiltrating cells and also in the necrotic areas and in some neighbouring hepatic cells. These appeared intact but were presumably damaged cells in the very early stages of necrosis.

**Immunofluorescence and Histological Examination of the Spleen**

- **Five Minutes.** No viral antigen could be detected.
- **Sixteen Hours.** Viral antigen was present in a small number of perifollicular cells, presumably macrophages.
- **Twenty-four Hours.** The number of infected cells in the red pulp, mainly perifollicular in distribution (Figure 7), had approximately doubled. Antigen could also be seen in the branching cytoplasm of occasional cells within the splenic follicles.
- **Thirty-two Hours.** A few infected lymphocytes were now present in the follicles, but most cells in the white pulp were still uninfected.
- **Forty-eight Hours.** Increased numbers of infected cells, mainly large mononuclears, were scattered throughout the red pulp and concentrated in perifollicular regions. Routine histological sections showed an increase
Figure 9. Spleen at four days after intravenous injection of $10^7 LD_{50}$ of WE$_3$ strain LCM virus. Marked infection in perifollicular region and relatively little in follicle. Fluorescent antibody stain. X 200.
in frequency of the localized collections of large pale mononuclear cells which are normally present at the edge of some follicles.

**Three Days.** More infected cells were present in the red pulp, mainly in perifollicular regions. Occasional infected megakaryocytes were now seen, with bright cytoplasmic and nuclear fluorescence. There was a marked increase in size of the collections of large pale mononuclear cells and some follicles were almost surrounded by collars of these cells (Figure 8).

**Four Days.** There was a further increase in infected cells, but in the white pulp still only a few lymphocytes and large branching cells were infected (Figure 9). Infection had now appeared in the endothelium and walls of blood vessels, including the central arterioles of the follicles. Histologically, even greater numbers of the large pale mononuclear cells could be seen. Small amounts of extracellular eosinophilic fibrinoid material were now present between these cells in some areas (see also Section VI). There was a marked increase in pyroninophilic cells, in both the red and the white pulp of the spleen.

**Five Days.** There appeared to be fewer infected cells in the spleen than at four days, but the predominantly perifollicular distribution of infected cells was
Figure 10. Spleen at five days after intravenous injection of $10^7 LD_50$ of WE$_3$ strain LCM virus. Viral antigen in and around necrotic follicle. Fluorescent antibody stain. X 200.
Figure 11. Spleen at five days after intravenous injection of $10^7$ LD$_{50}$ of WE$_3$ strain LCM virus. Necrotic changes in large pale cells in perifollicular region. Haematoxylin and eosin. X 250.
Figure 12. Spleen at five days after intravenous injection of $10^7LD_{50}$ of WE$_3$ strain LCM virus. Advanced necrotic changes in follicle. Cuff of intact cells around central arteriole. Haematoxylin and eosin. X 250.
Figure 13. Spleen at five days after intravenous injection of $10^7LD_{50}$ of WE$_3$ strain LCM virus. Immunoglobulin in perifollicular necrotic areas. Fluorescent antibody stain. X 125.
unchanged. Areas of necrosis could be seen in many perifollicular regions, occurring within foci of infected cells and containing numerous granules of viral antigen (Figure 10). In routine histological sections the necrotic areas were eosinophilic and homogeneous (Figure 11) and extended towards the centre of some follicles, which were almost devoid of cells (Figure 12). In the necrotic zones were scattered large mononuclear cells, some having pyknotic nuclei, and many of these cells were pyroninophilic. A cuff of cells, most of them pyroninophilic, usually remained around the central arteriole (Figure 12). There was an increase in lymphoid cells in the red pulp, with lessened distinction between red and white pulp.

Fluorescent antiserum against mouse immunoglobulin showed staining in the perifollicular necrotic areas and this was more marked around their edges and brightest in surrounding intact cells (Figure 13). In other areas there were perifollicular and intrafollicular collections of immunoglobulin-containing cells. Immunoglobulin was also present in the cytoplasm of large numbers of cells scattered throughout the red pulp, singly or in small groups. In normal spleens, in contrast, immunoglobulin was present in a much smaller number of cells,
Figure 14. Spleen at six days after intravenous injection of $10^7$LD$_{50}$ of WE$_3$ strain LCM virus. Advanced necrosis. Haematoxylin and eosin. X 40.
scattered in the red pulp. The specificity of staining for mouse immunoglobulin in the spleen was established in the same way as for that in the liver (see page 29).

Six Days. There was more extensive perifollicular and follicular necrosis, with greater deposition of eosinophilic material (Figure 14). Fewer intact infected cells were seen, and immunoglobulin staining in the necrotic areas had increased.

Thus, there is evidence that eosinophilic necrosis occurred in perifollicular collections of large mononuclear cells which stained for both LCM virus antigen and mouse immunoglobulin.

Immunofluorescence and Histological Examination of Other Organs

These were examined six days after the intravenous injection of WE$_3$ strain LCM virus. Widespread infection was present throughout all tissues examined, except in the heart, where no infection of the myocardium was detected. In almost all organs, the interstitial tissues showed a moderate to marked infiltration with mononuclear cells. Some of these cells were infected and some contained immunoglobulin. Normal control tissues showed fewer immunoglobulin-containing mononuclear cells.

The lungs showed a marked interstitial pneumonitis
Figure 15. Cervical lymph node at six days after intravenous injection of $10^7\text{LD}_{50}$ of WE$_3$ strain LCM virus. Advanced necrosis. Haematoxylin and eosin. X 40.
and moderate pleural effusions were present. The resulting interference with respiration no doubt contributed to the death of the mice. Scattered infected cells were present in the renal glomeruli, most of which contained small to moderate amounts of immunoglobulin. In control mice, small amounts of immunoglobulin were seen in occasional glomeruli only. The pancreas and omentum were oedematous. In the adrenals, viral antigen was present in approximately 80% of cells of the cortex and in a few cells of the medulla. The glands appeared histologically normal and no immunoglobulin was detected. Cortisone is known to exert a protective effect in LCM virus infection of mice (Hotchin & Cinitis, 1958; Mims, C.A., unpublished data). Perhaps the absence of mononuclear cell infiltration in the adrenals is due to the high local concentration of steroid hormones.

In the lymph nodes, the infected cells present were mainly large mononuclear cells with cytoplasmic processes. Histological examination showed gross depletion of lymphocytes, especially in central areas. Eosinophilic necrotic material and karyorrhectic debris were scattered throughout the nodes (Figure 15). In the thymus, numerous infected cells were present in the
Figure 17. Thymus at six days after intravenous injection of $10^7LD_{50}$ of WE$_3$ strain LCM virus. Cortico-medullary inversion. Haematoxylin and eosin. X 40.
medulla and some in the cortex (Figure 16). There was gross depletion of thymocytes in the cortex and a slight increase in the medulla, with reversal of the normal cortico-medullary appearances (Figure 17). Dilated blood vessels were present, mainly in the medulla, and karyorrhectic debris was scattered throughout, especially in the cortex. The changes in lymph nodes and thymus are discussed more fully in Section VI.

**Viral Replication Cycle Times in Infected Macrophages and Hepatic Cells**

Infected Kupffer cells appeared in the liver between nine and eleven hours after intravenous injection of the WE3 strain of LCM virus, whereas infected hepatic cells first appeared between eighteen and twenty-four hours. This suggests that infection may have been transmitted from Kupffer cells to hepatic cells. To indicate whether the replication cycle times of the virus in the two types of cells were consistent with this mechanism, the following experiments were carried out.

Ring cultures of mouse peritoneal macrophages were prepared and WE3 strain LCM virus was added at a multiplicity of 10LD50 per cell. Fluorescent antibody stains were done at two-hourly intervals. No infected cells were present at ten hours and new viral antigen was
first detected in a few cells at twelve hours after infection. At fourteen hours, a larger number of cells were infected. These times are consistent with the first detection of infected Kupffer cells in vivo at eleven hours after the intravenous injection of virus. Mims (1960b) has shown that retrograde injection into the common bile duct causes virus to enter directly into the cytoplasm of hepatic cells. An inoculum containing $10^7 LD_{50}$ of WE$_3$ strain LCM virus was injected up the common bile duct of each of seven mice. Twelve hours later, fluorescent antibody staining showed viral antigen in a few single hepatic cells. Occasional infected Kupffer cells and pancreatic acinar cells were also present, the latter indicating that some of the inoculum had passed along the pancreatic duct to enter the cytoplasm of acinar cells. After intravenous injection of virus, hepatic cells were not infected by eighteen hours, so it is unlikely that they were infected by the original inoculum. At twenty-four hours after injection into the bile duct, approximately twice as many infected hepatic cells were present as at twelve hours, but they were still single and no foci were seen. This indicates that there is an interval of more than twelve hours before spread of infection to adjacent hepatic cells.
Effect of Antiserum on the Development of Necrotic Lesions

The necrotic lesions which developed in the spleen and liver of mice four to five days after infection with the WE$_3$ strain of LCM virus, showed positive staining for mouse immunoglobulin. It was therefore of interest to determine whether the presence of antibody against LCM virus at an early stage of infection would accelerate the development of necrosis. Mice were inoculated intravenously with $10^7$ LD$_{50}$ of WE$_3$ strain LCM virus and at two and a half days, half received an intravenous injection of 1.0 ml of guinea pig hyperimmune antiserum against LCM virus. Both groups of mice were killed at three days after infection, but neither the antiserum-injected nor the control mice showed necrotic lesions.

That is, (heterologous) antiserum to LCM virus, in the presence of cells infected with the virus, did not by itself result in the development of necrosis.

B. Intravenous Injection of Armstrong Strain LCM Virus

When mice were injected intravenously with $10^5$ LD$_{50}$ of the mouse-adapted Armstrong strain of LCM virus, no infected cells could be detected in any tissues by daily fluorescent antibody staining up to eleven days after injection. The mice did not become sick and no
histological changes were seen. By titration of tissue specimens at five days, however, the virus proved to be present in very small amounts in the liver and spleen ($10^{3.5 \text{LD}_50 \text{ per gram}}$) and in the blood ($10^{2.5 \text{LD}_50 \text{ per ml}}$). That is, the Armstrong strain of LCM virus showed little or no replication in the liver and spleen after intravenous injection. It may be noted that the mice developed immunity to intracerebral challenge with either strain of LCM virus.

C. **Clearance of LCM Virus from the Blood**

The above experiments show that intravenously injected WE$_3$ strain LCM virus grows in the viscera of adult mice, whereas there is little or no growth of the mouse-adapted, Armstrong strain of virus. To test the possibility that Armstrong strain virus is not taken up from the blood of mice in significant amounts, the blood clearance of the two strains of virus was compared. Clearance of carrier virus, which is similar in its effects to Armstrong strain virus, was also tested. In addition, clearance in adult and infant mice was compared, in view of the differences found in the pathogenesis of LCM virus infection of the central nervous system (see Section V).
Table 2. CLEARANCE OF LCM VIRUS FROM THE BLOOD OF ADULT MICE AFTER INTRAVENOUS INJECTION.
Table 3. CLEARANCE OF LCM VIRUS FROM THE BLOOD OF MICE AFTER INTRAVENOUS INJECTION.
Adult Mice

Since guinea pig lung and mouse brain virus preparations may contain large amounts of tissue debris, in these clearance experiments infected mouse serum was used. Serum was collected from mice six days after intraperitoneal infection with $10^5 \text{LD}_{50}$ of WE$_3$ strain or Armstrong strain LCM virus, and from mice of an LCM virus carrier colony. The sera were titrated and inocula of 0.5 ml were injected intravenously into mice. Groups of three mice were killed at five minutes, thirty minutes and sixty minutes and their sera were titrated individually. The results of typical experiments are shown in Table 2. Armstrong strain virus and carrier virus were not cleared to a significant extent, whereas about ninety per cent of the WE$_3$ strain virus was rapidly removed from the blood. Similar results were obtained using a guinea pig lung preparation of WE$_3$ strain virus and a mouse brain preparation of Armstrong strain virus, as shown in Table 3, and with virus of both strains which had been passaged once in mouse embryo fibroblast tissue culture.

It was necessary to show that the clearance of WE$_3$ strain virus was not to a significant extent due to adsorption to the cellular elements of the blood. Heparinized blood was taken five minutes after the intravenous injection of virus and the plasma and cells were titrated separately. The cells were found to
contain only one-tenth as much virus as the plasma. Clearance could not therefore be explained by adsorption of virus to the cellular elements of the blood, and most of the virus had in fact been removed from the blood stream.

It was thought possible that the virus remaining at thirty and sixty minutes in the WE₃ strain experiment (Table 2) was not cleared because it consisted of mouse-adapted (Armstrong type) virus. Use was therefore made of the fact that mouse-adapted LCM virus is not lethal to guinea pigs by the intraperitoneal route. The thirty-minute serum specimen in the WE₃ strain experiment, representing the uncleared virus, was found to kill guinea pigs on intraperitoneal inoculation. When this serum was injected intravenously into mice, the subsequent thirty-minute serum was also lethal to guinea pigs. The uncleared virus, therefore, probably did not consist of mouse-adapted virus.

Infant Mice

Three-day-old mice were injected intravenously with WE₃ strain and Armstrong strain stock suspensions of LCM virus. Three mice were killed at thirty minutes and at sixty minutes and were bled from a renal artery rather than the heart, in case any of the inoculum had entered
the pleural cavity. Individual titration of the sera showed that neither Armstrong nor WE₃ strain virus was detectably cleared from the blood of the infant mice within sixty minutes. The results of typical experiments are shown in Table 3.

In summary, WE₃ strain LCM virus was rapidly cleared from the blood of adult mice, whereas mouse-adapted virus was not. In infant mice, however, neither WE₃ nor Armstrong strain virus underwent rapid clearance from the blood. Further experiments concerned with the clearance of LCM virus are reported in Section X.

DISCUSSION

After the intravenous injection of large particle colloids in the mouse, approximately ninety-five per cent of the particles are taken up by the liver, one to four per cent by the spleen and relatively little by other organs (Dobson et al., 1949). Mims (1959a) has shown that intravenously injected ectromelia virus is taken up by the organs of the mouse in much the same proportions as are inert particles. Within two minutes after intravenous injection, ectromelia virus, India ink (Mims, 1959a) and colloidal gold particles (Parks & Chiquoine, 1957) are being phagocytosed by reticuloendothelial cells in the mouse liver.
When a large dose of the WE₃ strain of LCM virus was injected intravenously into adult mice, virus was rapidly cleared from the blood, about ninety per cent disappearing by five minutes after inoculation. At this time, viral antigen could be detected in Kupffer cells in the liver. Although the inoculum contained $10^7 LD_{50}$ of virus and most of the virus particles would have been taken up by the Kupffer cells, of which there are approximately $10^7$ in the mouse liver, less than one per cent of these cells contained detectable viral antigen at five minutes. However, antigen in the inoculum would be phagocytosed to a varying extent by different Kupffer cells and small amounts of antigen in a cell would not be detectable by immunofluorescence. Parks (1956) has shown that different liver macrophages, whatever their position in the lobule, take up differing amounts of intravenously injected particles. By one hour after the intravenous injection of WE₃ strain LCM virus, antigen had disappeared from the Kupffer cells. Similar findings have been reported for ectromelia virus (Mims, 1959a), and it is assumed that by this time the Kupffer cells had degraded the LCM viral antigen. After eleven hours, infected Kupffer cells, containing new viral antigen, were seen in the liver. In infant mice, in contrast to adult
mice, intravenously inoculated \( \text{WE}_3 \) strain LCM virus was not cleared to a significant extent by sixty minutes. It has similarly been reported that infant mice fail to clear Sindbis virus from the blood as effectively as adults (Johnson & Mims, 1968).

Intravenously inoculated Armstrong strain LCM virus and carrier virus were not significantly cleared from the blood of adult mice, probably reflecting lack of uptake by liver macrophages. It has been shown that mouse peritoneal macrophages in vitro are infected to a much lesser extent by Armstrong strain virus than by \( \text{WE}_3 \) strain virus (see Section X). The failure to remove Armstrong strain virus from the blood is correlated with the virtual absence of visceral infection. There is also a failure to clear the mouse-adapted carrier strain of LCM virus from the blood, and presumably mouse adaptation has led to some alteration in the virus particles, affecting their recognition by the mouse as foreign material. Shwartzman (1946) showed that the adaptation of LCM virus to growth in mouse tissues causes a reduction in its lethality for adult mice by extraneurral inoculation (see also Section X).

When the \( \text{WE}_3 \) strain of LCM virus was injected into the common bile duct of mice, so having direct access
to hepatic cells, viral antigen appeared in the cells within twelve hours. After the intravenous injection of this virus, however, infected hepatic cells did not appear until between eighteen and twenty-four hours later. This would be an unduly long latent period if the hepatic cells had been infected at the time of inoculation. Thus, it appears that when virus in the blood enters the liver, it first infects Kupffer cells and spreads from these to hepatic cells. The uptake and growth of virulent viruses in liver macrophages, leading to the infection of hepatic cells, has been suggested for distemper virus in dogs (Coffin et al., 1953) and yellow fever virus in monkeys (Tigertt et al., 1960) and described for rabbitpox virus in rabbits (Mims, 1964a) and ectromelia (Mims, 1959b) and cowpox (Mims, 1968a) viruses in mice. In the spleen, after intravenous injection of LCM virus, the initial infection appeared in cells in perifollicular regions. It has been found that intravenously injected particulate materials localize mainly in large cells at the periphery of the follicles (Cappell, 1929; Odeblad et al., 1955), and following the intravenous injection of ectromelia virus, infection in the spleen first appeared in perifollicular cells (Mims, 1964a). Later, a few cells
within the splenic follicles became infected with LCM virus. Galindo & Imaeda (1962) have shown that macrophages are present in the white pulp of the mouse spleen.

The number of single infected hepatic cells at twenty-four hours after injection was similar to the number of Kupffer cells in which infection first appeared after the eclipse period, suggesting that each infected Kupffer cell infects only one or two hepatic cells. That is, the mode of spread of LCM virus from Kupffer to hepatic cells is more in keeping with localized or contact spread. Histological studies have shown that in the mouse liver there are about two Kupffer cells to every three hepatic cells (Edwards & Klein, 1961), so that each macrophage is probably in contact with only a small number of hepatic cells. The number of infected hepatic cells increased slowly between one and three days. The presence of increasing numbers of single infected hepatic cells as well as foci of increasing size, is evidence for both the continuing transmission of infection from Kupffer cells to hepatic cells and the spread of infection from these to adjacent hepatic cells.

Between three and four days after injection of virus, there was a marked increase in hepatic cell infection.
One factor contributing towards this delayed and relatively sudden increase may be a delay in the transmission of infection from infected hepatic cells to adjacent cells. This is suggested by the fact that when WE₃ strain LCM virus was injected into the common bile duct, single infected hepatic cells were seen at twelve hours and after twenty-four hours the infected hepatic cells present were still single. Also, after intravenous injection of the virus, hepatic cells became infected between eighteen and twenty-four hours and were still single at thirty-two hours; by forty hours, some pairs of infected cells were present. This slow spread of infection between hepatic cells may account for the lapse of several days before large numbers of infected cells became manifest.

The proportions of infected cells in the liver and spleen reached a maximum at four days and then appeared to decline, at the time of development of necrotic changes. Since the necrosis occurred within foci of infected cells, this may account for some decrease in the number of intact infected cells. Also, at about this time, the spread of infection may have begun to be controlled. Antibodies produced against LCM virus may have reached effective levels in the tissues and in fact, immunoglobulin could be seen in and around the necrotic lesions, by immunofluorescence
staining. Infiltrating mononuclear cells could play a part in controlling the spread of infection, although the mechanism of their activities is not clear. Finally, although LCM virus infection does not appear to give rise to the production of interferon (Wagner & Snyder, 1962; Mims and Subrahmanyan, 1966), interferon-like substances (Veltri & Kirk, 1969) may be concerned in control of the spread of infection.

Erythrocytes were seen in some of the necrotic foci, probably due to blood from sinusoids coming to occupy the spaces left by hepatic cells which had disintegrated. Similar but more extensive "blood lakes" occur in the liver of mice infected with Rift Valley fever virus (Mims, 1957a). The cell debris seen in the lumina of veins in the liver may have arisen from degenerating hepatic cells or mononuclear cells. Degenerate hepatic cells have been observed lying free in blood vessels in the liver of monkeys infected with yellow fever virus (Cowdry & Kitchen, 1930). In mice infected with Rift Valley fever virus (Mims, 1957a) or ectromelia virus (Mims, 1959b), cell fragments could be detected in blood smears as well as in hepatic blood vessels.

The observation that necrosis of infected hepatic
cells was associated with the development of an infiltration of mononuclear cells, some of which surrounded the necrotic areas, suggests that the necrosis may result from an interaction between sensitized mononuclear cells and tissue cells which contain large amounts of viral antigen. The in vitro experiments of Lundstedt (1969) suggest that sensitized lymphoid cells may have a cytopathic effect on cells infected with LCM virus. The hepatic cells which contained immunoglobulin were probably injured infected cells which, though still histologically normal, had undergone sufficient change to allow the entry of antibody against LCM virus.

In the spleen, also, necrotic lesions were associated with the presence of both infected mononuclear cells and immunoglobulin. Several lines of evidence suggest that the presence of immunoglobulin in the necrotic hepatic cells and perifollicular splenic lesions is a secondary phenomenon; circulating antibody to LCM virus is probably deposited in dead or dying infected cells. The presence of (heterologous) antibody in association with infected tissues does not by itself cause pathological lesions, as demonstrated by the administration of antiserum to acutely infected mice. This is also shown by the
situation in adoptively immunized LCM virus carrier mice (Volkert et al., 1964; Section VII), which did not develop pathological lesions despite the production of high levels of antibody to LCM virus.

In LCM virus carrier mice, there is evidence for the presence of circulating virus-antibody complexes (Section VII; Oldstone & Dixon, 1969). If such complexes also occur in adult mice infected with LCM virus, their deposition in the tissues may contribute to the development of the necrotic lesions. It has been shown that the deposition or formation of immune complexes in hepatic sinusoids may result in focal necrosis of liver cells (Hartley & Lushbaugh, 1942; Steiner, 1961), and that localization of immune complexes in areas of minimal hepatocellular damage may cause magnification of the liver damage (Paronetto & Popper, 1965). Also, in mice infected with LCM virus, which infects reticuloendothelial cells, there is an impairment of phagocytic capacity (Gledhill et al., 1965). Perhaps this may favour the deposition of virus-antibody complexes in the tissues, since immune complexes are normally cleared from the blood by the reticuloendothelial system (Benacerraf et al., 1959).

Thus, it appears that cellular and possibly humoral immune responses are involved in the development
of necrotic lesions in the tissues of mice infected with LCM virus. These lesions vary in different strains of mice (see Section XI) and their pathogenesis in the lymphoid tissues and liver is described more fully in Section VI.

SUMMARY

The initiation and development of infection in the visceral organs of mice after intravenous injection of a lethal dose of LCM virus has been analysed by the immunofluorescence technique, in association with histological studies.

In the liver, virus was taken up within a few minutes by the Kupffer cells. Viral replication first occurred within these cells and infection spread from them to the hepatic cells. In the spleen, infection first occurred in perifollicular regions and later throughout the red pulp and in some cells of the white pulp. Infiltration of the tissues by mononuclear cells, necrotic changes in the liver and spleen and signs of sickness all developed at about the same time. Necrosis occurred in foci of infected cells and immunoglobulin was present in and around the lesions.

The mouse-adapted strain of LCM virus was not cleared from the blood to a significant extent and infection of the tissues was not detectable by immunofluorescence.
V. THE RESPONSE OF MICE TO THE INTRAVENOUS INJECTION OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS

2. INFECTION OF THE CENTRAL NERVOUS SYSTEM

Lymphocytic choriomeningitis virus was so named because, on intracerebral inoculation into animals, it caused a mononuclear cell infiltration which was limited to the meninges and choroid plexuses (Armstrong & Lillie, 1934). The histological changes in mice infected with LCM virus have been described by Traub (1936 a) and Lillie and Armstrong (1945).

Viruses which infect the central nervous system (CNS) usually undergo an initial stage of visceral infection which gives rise to a viraemia. The mechanisms of haematogenous spread to the CNS by viruses which cause encephalitis, such as herpes simplex virus and various arboviruses, have been investigated in detail and are reviewed by Johnson and Mims (1968). However, the frequency of occurrence of viral meningitis far exceeds that of encephalitis and the latter may in some cases be a late development in a meningitic illness. There has nevertheless been a lack of studies of the transmission
of viruses across the blood-cerebrospinal fluid junction. This section describes the development of CNS infection in mice after the intravenous injection of LCM virus, as discerned by immunofluorescence and histological examination.

RESULTS

A. Injection of Adult Mice

A large dose \((10^7 \text{LD}_{50})\) of the WE_3 strain of LCM virus was injected intravenously into adult WEHI mice. This dose causes 100% lethality by eight days. Clinico-pathological observations have been described in Section IV, and it may be noted that mice failed to show the CNS signs seen after intracerebral inoculation. At daily intervals after infection, four mice were killed and the brains removed for immunofluorescence and histological studies. Heparinized blood samples were tested daily for infectivity by intracerebral inoculation into adult mice. Viraemia was found to be present from the first day.

Fluorescence Microscopy and Histology of the Brain and Meninges

Four Days. LCM viral antigen was first detected in the CNS at this time. Antigen was present in the walls of a moderate number of blood vessels in the meninges and in
Figure 18. Brain substance at four days after intravenous injection of $10^7LD_{50}$ of WE$_3$ strain LCM virus. Infected blood vessels. Fluorescent antibody stain.  X 300.
Figure 19. Meninges at four days after intravenous injection of $10^7LD_{50}$ of WE$_3$ strain LCM virus. Infected meningeal cells. Fluorescent antibody stain. X 300.
Figure 20. Choroid plexus at four days after intravenous injection of $10^7LD_{50}$ of WE$_3$ strain LCM virus. Infected cells in core of choroid plexus. Fluorescent antibody stain. X 400.
Figure 21. Meninges at five days after intravenous injection of $10^7LD_{50}$ of WE$_3$ strain LCM virus. Infection in meninges and Virchow-Robin space. Fluorescent antibody stain. X 300.
Figure 22. Choroid plexus at five days after intravenous injection of $10^7LD_{50}$ of $WE_3$ strain LCM virus. Infection of choroid plexus epithelial cells. Fluorescent antibody stain. X 200.
Figure 23. Meninges at five days after intravenous injection of $10^7 \text{LD}_{50}$ of WE$_3$ strain LCM virus. Mononuclear cell infiltration in meninges and Virchow-Robin space. Haematoxylin and eosin. X 400.
the brain substance (Figure 18). Some infected meningeal cells were present, usually adjacent to blood vessels (Figure 19), and there were foci of infected cells in the cores of choroid plexuses (Figure 20). The mice were well at this stage and no changes were detectable in haematoxylin and eosin stained sections.

**Five Days.** The mice had become sick and the infection was more widespread. There was now marked infection in the meninges, and infected cells were seen in some Virchow-Robin spaces (Figure 21). Infection was also present in the walls of many cerebral blood vessels. There were more infected cells in the cores of choroid plexuses than at four days and antigen was now seen in small numbers of ependymal cells and in some choroid plexus epithelial cells (Figure 22). Occasional subependymal and submeningeal cells were infected. Routine histological sections now showed a moderate cellular infiltration of the meninges, Virchow-Robin spaces, subependymal regions and cores of choroid plexuses. The infiltrating cells (Figure 23) were mostly large, pale mononuclears, with some small lymphocytes and occasional plasma cells and polymorphonuclear leukocytes. A few of these cells contained mouse immunoglobulin, as detected by immunofluorescence staining.
Six Days. Some mice had died and most of the survivors were sick. Viral antigen was now widespread in meninges, blood vessel walls and cells in Virchow-Robin spaces, and most ependymal and choroid plexus epithelial cells were infected. In infected blood vessels, it was not possible to determine whether the infection was confined to the endothelial cells alone. More infected cells were present in the brain substance than at five days, but they were still limited to subependymal and submeningeal areas. The mononuclear cell infiltration had increased and immunoglobulin was present in many of these cells.

Seven Days. Most mice had died by this time and the survivors were all sick. There had been a further increase in the numbers of infected cells, but the distribution was unchanged and infection of cerebral tissue was still confined to submeningeal and subependymal areas. Histological changes were not detectably different from those at six days.

In these intravenously injected mice, cerebral blood vessels became infected, but the virus did not spread into the brain substance. To determine whether WE<sub>3</sub> strain LCM virus could spread through the cerebral tissue after direct inoculation into the brain, mice were injected intracerebrally with $10^7LD_{50}$ of virus. Immunofluorescence examination of the brains at six days showed
Figure 24. Brain and meninges at six days after intracerebral injection of $10^7$LD$_{50}$ of WE$_3$ strain LCM virus. Infection confined to the meninges. Fluorescent antibody stain. X 125.
that infection again remained largely confined to the meninges, choroid plexuses and ependyma (see also Section X; Wilsnack & Rowe, 1964), and had not extended into the brain substance (Figure 24).

In other experiments, groups of adult mice were injected intravenously with a smaller dose of WE₃ strain virus (10³LD₅₀) or with a large dose (10⁷LD₅₀) intraperitoneally or subcutaneously into the footpad. The brains were examined at seven days, the mice inoculated intraperitoneally being sick, but no infected cells or histological changes were detected. Evidence has been obtained which suggests that intraperitoneally injected WE₃ strain LCM virus multiplies in the brain only when the brain is damaged at the time of inoculation (Lehmann-Grube, 1964 a). Other mice were injected intravenously with 10⁵LD₅₀ of the Armstrong strain of LCM virus, which grows in the CNS after intracerebral inoculation (Section X; Lehmann-Grube, 1964 a). However, the mice remained well and no infected cells or histological changes were detected in the CNS up to eleven days after injection.

Since viral antigen was first detected in the CNS at four days after intravenous injection with a large dose of WE₃ strain virus, but not with Armstrong strain virus, it was of interest to compare the extent of virus
Table 4. Virus titres in liver and blood of adult mice at three days after intravenous injection of $10^{5}\text{LD}_{50}$ of different strains of LCM virus.

<table>
<thead>
<tr>
<th>STRAIN OF VIRUS</th>
<th>LIVER TITRE  $(\log_{10}\text{LD}_{50} \text{ per gram})$</th>
<th>BLOOD TITRE  $(\log_{10}\text{LD}_{50} \text{ per ml.})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WE$_3$</td>
<td>7.3</td>
<td>5.4</td>
</tr>
<tr>
<td>ARMSTRONG</td>
<td>2.3</td>
<td>1.8</td>
</tr>
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</table>
growth in the viscera and the levels of virus in the blood. Therefore, groups of six mice were injected intravenously with $10^5 \text{LD}_{50}$ of WE$_3$ or Armstrong strain virus and at three days, pooled livers and blood were titrated. The results are shown in Table 4, and it can be seen that WE$_3$ strain virus was present in high titres in the liver and blood, whereas little or no growth of Armstrong strain virus had occurred.

In summary, invasion of the CNS of adult mice by LCM virus occurred only when large doses of virus of the WE$_3$ strain were injected intravenously. The first infected cells, seen at four days, were associated with small blood vessels, principally in the meninges and also in the choroid plexuses and brain substance.

B. Infection of Infant Mice

It has generally been found that extraneurally inoculated neurotropic viruses invade the CNS more readily in infant mice than in adult mice (Sigel, 1952). Four-day-old mice were injected intraperitoneally with $10^5 \text{LD}_{50}$ of the WE$_3$ strain of LCM virus. At daily intervals, heparinized blood samples were tested for infectivity and the virus titres of pools of six brains were determined. Viraemia was found to be present from the first day, as in adult mice, and the brain virus
Figure 25. Brain virus titres in infant mice after intraperitoneal infection with $10^5LD_{50}$ of WE$_3$ strain LCM virus.
titres are shown in Figure 25. The virus titres in the brain increased rapidly during the first two days and became maximal at five days. The mice remained well until six days after injection, when some became sick, with trembling, laboured respiration and reduced activity. They were found to have pleural and peritoneal effusions. Deaths occurred from nine to eighteen days after infection and approximately one-third of the mice survived. This is in contrast to the uniform mortality seen after the intraperitoneal injection of an equivalent dose in adult mice (see Section X) and can be explained by the lesser degree of development of the immune response to LCM virus infection in infant mice (Hotchin & Weigand, 1961a).

At intervals after infection, four mice were killed and the brains (intact in the skull, with skin removed) were taken for immunofluorescence examination. To aid identification of small blood vessels in the CNS, India ink (Pelikan, Gunther Wagner, Hanover) was injected into the heart just before death.

Fluorescence Microscopy of the Brain and Meninges
Eighteen Hours. A small number (1-2 per brain section) of infected large mononuclear cells were present in the subarachnoid space, apparently free in the cerebrospinal fluid (CSF). There was no viral antigen elsewhere in the
Figure 26. Brain substance of infant mouse at twenty-four hours after intraperitoneal injection of $10^5 LD_{50}$ of WE$_3$ strain LCM virus. Infection in blood vessel walls and in adjacent brain substance. Fluorescent antibody stain. X 600.
brain or meninges. In smears of heart and tail blood, occasional infected large white blood cells were seen, and these cells had a similar appearance, under fluorescence or phase microscopy, as the infected cells present in the subarachnoid space. They were about 15 microns in diameter, with a bilobed nucleus and a large amount of foamy cytoplasm, and both nucleus and cytoplasm contained viral antigen. Cells of similar morphology were seen in blood smears of normal four-day-old mice, but they did not stain with fluorescent antibody.

Twenty-four Hours. As well as in mononuclear cells in the subarachnoid space, viral antigen was now seen in the walls of a moderate number of blood vessels in the meninges, choroid plexuses and brain substance (Figure 26). Occasional infected cells were present around small blood vessels in the meninges and brain substance (Figure 26).

Forty-eight Hours. Viral antigen was now present in moderate numbers of meningeal cells, mainly adjacent to blood vessels, and in a few ependymal cells and choroid plexus epithelial cells. There was some increase in the number of infected blood vessels and in the number of infected cells in the brain substance.

Three Days. By this time, infection was widespread in the meninges and throughout the brain, especially in the
Figure 27. Brain of infant mouse at three days after intraperitoneal injection of $10^5LD_{50}$ of WE$_3$ strain LCM virus. Extensive infection in cerebellum and in grey matter of brain. Fluorescent antibody stain. X 200.
Figure 28. Ventricle of infant mouse at three days after intraperitoneal injection of $10^5 \text{LD}_{50}$ of WE$_3$ strain LCM virus. Extensive infection in choroid plexus epithelial cells, ependyma and brain substance. Fluorescent antibody stain. X 80.
gray matter and in the cerebellum (Figure 27). Antigen was now present in many ependymal and choroid plexus epithelial cells (Figure 28).

**Four Days.** A general increase had occurred in the number of infected cells throughout the brain and meninges.

**Five Days to Seven Days.** The appearances by immunofluorescence remained unchanged from those seen at four days. However, titration methods showed that there was a marked increase in infectious virus in the brain between four and five days (Figure 25).

In other experiments, groups of four-day-old mice were injected intraperitoneally with a small dose \((10^2\text{LD}_{50})\) or subcutaneously with a large dose \((10^5\text{LD}_{50})\) of WE\(_3\) strain LCM virus. Immunofluorescence examination of the CNS at seven days showed identical appearances to those seen at the same time after intraperitoneal injection of the large dose of virus. The liver and spleen also showed marked infection at seven days, in all cases.

Thus, after extraneural inoculation in infant mice, the WE\(_3\) strain of LCM virus invaded the CNS much earlier than in adult mice and spread rapidly throughout the meninges and brain substance. Even a small dose of virus in infant mice resulted in CNS infection.
<table>
<thead>
<tr>
<th>STRAIN OF VIRUS</th>
<th>LIVER TITRE ( \log_{10} \text{LD}_{50} ) per gram</th>
<th>BLOOD TITRE ( \log_{10} \text{LD}_{50} ) per ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>WE₃</td>
<td>7.3</td>
<td>6.4</td>
</tr>
<tr>
<td>ARMSTRONG</td>
<td>4.7</td>
<td>4.1</td>
</tr>
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</table>

Table 5. VIRUS TITRES IN LIVER AND BLOOD OF INFANT MICE AT THREE DAYS AFTER INTRAPERITONEAL INJECTION OF \( 10^5 \text{LD}_{50} \) OF DIFFERENT STRAINS OF LCM VIRUS.
Other four-day-old mice were injected intraperitoneally with $10^5 \text{LD}_{50}$ of the Armstrong strain of LCM virus. At seven days, a moderate infection of the liver and spleen had occurred. In the CNS, infection was present in the meninges, choroid plexus epithelium and ependyma and also in subependymal and submeningeal cells. However, no infected cells were detected in cerebral blood vessels or in the brain substance. Thus, the Armstrong strain of LCM virus invaded the CNS after extraneural inoculation in infant mice, whereas it failed to do so in adult mice. It did not give rise to infection in the brain substance by seven days, in contrast to WE$_3$ strain LCM virus, which had spread throughout the CNS by this time.

Since the extent of visceral growth and the level of the resultant viraemia may be important in the spread of virus to the brain, a comparison was made of liver and blood titres three days after infection. The results, obtained with pooled specimens from six mice, are shown in Table 5, and it can be seen that the titres were much greater in the case of WE$_3$ strain virus infection.

To determine if newborn mice also showed this difference in infection with the two strains of LCM virus, mice were injected intraperitoneally, within twenty-four
<table>
<thead>
<tr>
<th>STRAIN OF VIRUS</th>
<th>BRAIN VIRUS TITRE (10^g_{10}LD_{50} per gram)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>INTRAPERITONEAL INJECTION</td>
</tr>
<tr>
<td>WE_3</td>
<td>8.6</td>
</tr>
<tr>
<td>ARMSTRONG</td>
<td>6.3</td>
</tr>
</tbody>
</table>

Table 6. BRAIN VIRUS TITRES IN INFANT MICE AT FIVE DAYS AFTER INTRAPERITONEAL OR INTRACEREBRAL INJECTION OF 10^5LD_{50} OF DIFFERENT STRAINS OF LCM VIRUS.
hours after birth, with $10^5 \text{LD}_{50}$ of the WE$_3$ or the Armstrong strain virus. The brains were titrated at five days (Table 6) and it was found that WE$_3$ strain virus was present in a much higher titre than Armstrong strain virus. Immunofluorescence staining at seven days showed the same difference in the pattern of CNS infection as seen at the same time after injection of four-day-old mice. That is, infection with Armstrong strain virus was virtually confined to the meninges, choroid plexuses and ependyma. By three weeks after intraperitoneal injection, however, the Armstrong strain virus had spread throughout the brain substance, to the same extent as the WE$_3$ strain virus. In contrast, after direct intracerebral injection of mice, less than twenty-four hours old, with $10^5 \text{LD}_{50}$ of either strain of virus, titration of the brains at five days (Table 6) showed that the Armstrong strain virus had grown in the brain to the same titre as the WE$_3$ strain virus (see also Section X). Immunofluorescence staining at seven days after intracerebral injection showed that with both strains of virus, infection was present throughout the meninges and brain substance.

In summary, in infant mice, extraneurally inoculated WE$_3$ strain LCM virus infected the CNS more
readily and at a much earlier stage than in adult mice. The initial entry of infection into the CNS appeared to be via the CSF spaces, and virus also spread into the brain substance from cerebral blood vessels. Extraneurally inoculated Armstrong strain virus readily infected the meninges in infant mice, though it did not cause CNS infection in adult mice. The brain substance was infected much more rapidly by the WE₃ strain virus than by the Armstrong strain virus.

DISCUSSION

Viruses may reach the CNS by way of the blood, peripheral nerves or olfactory channels. Although LCM virus may be able to spread to the CNS by the neural or olfactory routes, it is only the haematogenous route that has been investigated here. Before infection of the CNS by the haematogenous route occurs, there is usually a preliminary visceral phase of growth (Mims, 1957 b; Wenner and Kamitsuka, 1957; Johnson, 1964 b; 1965). After intravenous injection into adult mice, the WE₃ strain of LCM virus is rapidly cleared from the blood by phagocytic cells, mainly in the liver, and growth of virus then occurs in the liver, the spleen and elsewhere (see Section IV). Virus was shown to be
present in high titres in the blood and liver by three days (Table 4), and viral antigen was first detected in the CNS after four days.

After intraperitoneal injection of adult mice with a large dose of WE₃ strain LCM virus, Lehmann-Grube (1964a), using titration methods, first detected virus in the brain at four days. The rise in blood virus titre was shown to precede the increase in brain virus titre by one day. The magnitude, type and duration of viraemia may be critical in determining whether or not virus gains access to the CNS. In adult mice, perhaps it is only with a large dose of WE₃ strain virus that visceral growth is great enough to maintain a viraemia of sufficient magnitude to enable access to the CNS. The Armstrong strain of LCM virus, which is present at only low levels in the blood and tissues after extraneural inoculation (Table 4; Lehmann-Grube, 1964a; Section IV), failed to invade the CNS after intravenous inoculation.

In comparison with adult mice, infant mice showed a greater susceptibility to the development of CNS infection after extraneural inoculation with WE₃ strain LCM virus. This difference in susceptibility has also been noted with other neurotropic viruses (Bugher, 1941; Lennette and Koprowski, 1944; MacDonald, 1952)
and has been attributed to inadequacy of the viraemia in adult mice (Mims, 1957b). It has been shown that infant mice fail to clear the WE_3 strain of LCM virus from the blood as effectively as adults (see Section IV), so that a sustained viraemia is more readily attained, and this may contribute to the earlier entry of virus into the CNS in infant mice. A similar difference in blood clearance in infant and adult mice has been reported for Sindbis virus (Johnson & Mims, 1968). However, there may also be differences at the cellular level, preventing transmission of infection from the blood to the CNS (Sabin & Olitsky, 1938; Hurst, 1950; Johnson, 1964b).

The first appearance of LCM virus antigen in the CNS of infant mice was in large mononuclear cells free in the CSF, and these cells had a similar appearance to the infected mononuclear cells present in the blood at an early stage. It has been shown that LCM virus infects white blood cells (Baratawidjaja et al., 1965; Mims, 1966a; Section VII), but it is not known whether these cells may enter the CSF. It is possible that the actively phagocytic mononuclear Kolmer cells of the ventricular system (Kappers, 1953) might be of significance in the transmission of viruses into the CSF spaces, since there is some evidence that these cells reach the CSF by
migrating from the blood vessels and through the epithelium of the choroid plexuses (Carpenter, S. J., unpublished observations).

Virus free in the blood may enter the CSF by either passing or growing through meningeal capillaries or through the capillaries and epithelium of the choroid plexuses. Pappas & Tennyson (1962) demonstrated that colloidal particles injected intravenously into rabbits passed through choroid plexus capillaries into the surrounding connective tissue; the meningeal blood vessels, however, were not investigated. Mims (1960 b) obtained suggestive evidence in infant mice that neuroadapted strains of influenza virus may spread from the blood directly into the CSF. The potential for leakage from capillaries into the CSF was shown by Schultz & Frohlich (1965), who injected large quantities of bacteriophage intravenously into dogs and recovered small amounts from the CSF. There is some evidence that Japanese encephalitis virus may enter the CSF by growth through the choroid plexuses (Hamashima et al., 1959). Once virus particles have entered the CSF, they are rapidly distributed throughout the CSF spaces (Mims, 1960 a).

After the intravenous injection of WE3 strain
LCM virus in adult mice, infected cells first appeared in and around small blood vessels in the meninges and choroid plexuses. This indicates that virus infected small blood vessels and passed through their walls into surrounding tissues. Passage through meningeal blood vessels would enable virus to enter the CSF, but after passing through blood vessels of the choroid plexuses, virus is still separated from the CSF by the choroid plexus epithelium. Infected choroid plexus epithelial cells, however, were not seen until a day later, at the same time as infected ependymal cells appeared. By this time, a mononuclear cell infiltration into the meninges could be detected histologically. It would seem, therefore, that virus was already present in the CSF before infection of choroid plexus epithelium occurred and that it reached the CSF from sites of growth in meningeal blood vessels.

Circulating virus may spread to the CNS by passage or growth through small blood vessels within the brain substance. Macromolecules of ferritin have been shown to pass directly from the blood into the brain (Bondareff, 1964) and it is possible that this may occur in infection with tick-borne encephalitis virus (Albrecht, 1960, 1962). Histological evidence for the growth of
viruses in cerebrovascular endothelial cells preceding infection of the surrounding glial cells and neurones has been reviewed by Bang and Luttrell (1961). Immuno-fluorescence studies have provided evidence for viral growth in the endothelium of small cerebral blood vessels in canine distemper (Coffin & Liu, 1957), influenza (Hook et al., 1962), West Nile (Kundin et al., 1963), herpes simplex and Sindbis (Johnson, 1964 a, 1965) virus infections. After intravenous injection of the WE₃ strain of LCM virus in adult mice, there was widespread infection of cerebral blood vessels, but the infection did not spread to the surrounding brain substance. In contrast, when infant mice were injected extraneurally, infection spread readily from cerebral blood vessels into the brain substance. There are a number of possible sites of impedance to the spread of infection from cerebrovascular endothelium in adult mice. Maynard et al., (1957) have shown in the rat that the end-feet of astrocytes are the predominant structures that envelop the capillaries of the brain substance. To infect neurones, virus from infected cerebrovascular endothelial cells would have to pass through the capillary basement membrane to the applied glial dendritic end-feet and thence to the neurones with which the glial cells are in contact. Alterations in the capillary
basement membrane or in the astrocytic processes, for example, might account for the reduction in the ease of spread of infection in adult mice. Gradual changes have been observed in the cerebrovascular capillaries of rats, with the increase in age of young animals towards maturity (Donahue & Pappas, 1961), including a progressive thickening and increase in density of the basement membrane. However, even if LCM virus could enter glial cells from the cerebral blood vessels, contiguous spread of infection to other cells might not be able to occur in adult mice. This could account for the observation that even when LCM virus was injected directly into the brain of adult mice, infection did not spread through the brain substance. After intravenous or intracerebral injection, the only significant infection which developed in the brain substance was in cells in the subependymal and submeningeal regions. There is some evidence that subependymal cells, though histologically indistinguishable from cells in other areas of the brain, may comprise a functionally distinct cell population (Messier et al., 1958).

When infant mice were injected extraneurally with WE₃ strain LCM virus, infection developed rapidly in the meninges and brain substance and by three days, infection had spread throughout the CNS. After injection
of Armstrong strain virus, however, by seven days the infection was still confined to the meninges, choroid plexuses, ependyma and subjacent cerebral cells. There was no infection of cerebral blood vessels, and possibly as a result of this, the virus had not spread into the brain substance. Infection of the cerebrovascular endothelium may depend on the magnitude of the viraemia, and this was shown to be much greater at three days after infection with WE₃ strain virus than after infection with Armstrong strain virus (Table 5). The level of viraemia, in turn, is a function of the extent of virus growth in the viscera, and the virus titre in the liver at three days was much less in the case of Armstrong strain virus infection (Table 5). With the passage of time, however, these differences were no longer significant and by three weeks after infection, the Armstrong strain virus had spread throughout the brain substance, to the same extent as the WE₃ strain virus.

These experiments show that when adult mice are injected extraneurally with LCM virus, the initial route of entry of virus from the blood into the CNS is by infection of meningeal blood vessels and adjacent meningeal cells, whereby the CSF becomes infected. Spread of virus into the CSF through the choroid plexuses
occurs at a later stage, but virus does not spread from infected cerebral blood vessels into the brain substance. In infant mice, the initial spread of LCM virus into the CNS is by infection of the CSF and lining membranes, and infection also spreads from cerebral blood vessels into the brain substance.

SUMMARY

The development of infection in the central nervous system of adult mice, after the intravenous injection of LCM virus, has been studied by the immuno-fluorescence technique and correlated with histological changes. The WE₃ strain of LCM virus infected the walls of blood vessels in the CNS and passed through the meningeal vessels into the surrounding tissues and into the CSF. Spread of infection from the choroid plexuses occurred at a later stage, but infection was not transmitted from cerebral blood vessels to cells in the brain substance.

In infant mice inoculated extraneurally with WE₃ strain LCM virus, invasion of the CNS occurred much earlier and more readily than in adult mice and there was widespread infection of the brain substance as well as
the meninges. Although the Armstrong strain of LCM virus did not invade the CNS after extraneural inoculation in adult mice, it did so in infant mice.

Since its discovery in 1934 (Armstrong & Lillie), there have been many accounts of the pathological lesions produced by LCM virus in infected animals, including mice (Traub, 1936 a; Findlay & Stern, 1936; Lillie & Armstrong, 1945). In mouse cells the infection is basically non-cytopathic (Hotchin & Cimita, 1959; Benda & Cimiti, 1962) and immune cellular responses are thought to play an essential role in the production of lesions (Hotchin, 1962 a). Hyperplastic changes have been described in lymphoid tissue and Lillie & Armstrong (1945) also referred to a "peri-follicular hyaline thrombosis and haemorrhage" in the spleen and "necrotic changes in pulp and sinuses" of lymph nodes. Destruction of lymphocytes may occur in the spleen, lymph nodes and thymus of infected mice (Mansaka et al., 1969). In addition, focal necrosis of liver cells has been reported (Traub, 1936 a; Lillie & Armstrong, 1945; Collins et al., 1961) and Traub (1936 a) noted that this occurred in the immediate vicinity of collections of infiltrating mononuclear cells.
VI. THE PATHOGENESIS OF LESIONS IN THE LYMPHOID TISSUES AND LIVER OF MICE INFECTED WITH LYMPHOCYTIC CHORIOMENINGITIS VIRUS.

Since its discovery in 1934 (Armstrong & Lillie), there have been many accounts of the pathological lesions produced by LCM virus in infected animals, including mice (Traub, 1936 a; Findlay & Stern, 1936; Lillie & Armstrong, 1945). In mouse cells the infection is basically non-cytopathic (Hotchin & Cinitis, 1958; Benda & Činatl, 1962) and immune cellular responses are thought to play an essential role in the production of lesions (Hotchin, 1962 a). Hyperplastic changes have been described in lymphoid tissue and Lillie & Armstrong (1945) also referred to a "peri-follicular hyaline thrombosis and haemorrhage" in the spleen and "necrotic changes in pulp and sinuses" of lymph nodes. Destruction of lymphocytes may occur in the spleen, lymph nodes and thymus of infected mice (Hanaoka et al., 1969). In addition, focal necrosis of liver cells has been reported (Traub, 1936 a; Lillie & Armstrong, 1945; Collins et al., 1961) and Traub (1936 a) noted that this occurred in the immediate vicinity of collections of infiltrating mononuclear cells.
Figure 29. Spleen at forty-eight hours after intravenous injection of $10^5LD_{50}$ of WE$_3$ strain LCM virus. Most infected cells are perifollicular in distribution. Fluorescent antibody stain. X 200.
Figure 30. Spleen at three days after intravenous injection of $10^5LD_{50}$ of WE$_3$ strain LCM virus. Large pale mononuclear cells in perifollicular region. Haematoxylin and eosin. X 200.
When WEHI mice were infected with the WE₃ strain of LCM virus, gross lesions were observed in lymphoid tissues and focal necrosis occurred in the liver (see Section IV). Further studies of the nature and pathogenesis of these lesions were made and are reported here.

RESULTS

Spleen Lesions in Mice Infected with LCM Virus

WEHI mice were injected intravenously with $10^5 LD_{50}$ of the WE₃ strain of LCM virus. Spleens from three mice were taken daily for virus assay and for immunofluorescence and histological examination.

At twenty-four hours, there were infected large mononuclear cells, presumably macrophages, in the red pulp and perifollicular regions. More cells were infected by forty-eight hours, some of them in follicles (Figure 29) and there had been a further increase by three days. At three days, spleens were enlarged and there were collections of large pale mononuclear cells, some of them pyroninophilic and some of them infected, around follicles (Figure 30). Scattered throughout the spleen were occasional cells containing mouse immunoglobulin, and there had been a
Figure 31. Spleen at four days after intravenous injection of $10^5 \text{LD}_{50}$ of WE$_3$ strain LCM virus. Early necrotic changes in large pale cells in perifollicular region. Haematoxylin and eosin. X 200.
**Figure 32.** Spleen at five days after intravenous injection of $10^5\text{LD}_{50}$ of WE$_3$ strain LCM virus. More advanced necrotic changes in perifollicular region. Haematoxylin and eosin. X 200.
Figure 33. Spleen at five days after intravenous injection of $10^5 \text{LD}_{50}$ of WE3 strain LCM virus. LCM virus antigen in necrotic zone between follicles. Fluorescent antibody stain. X 300.
Figure 34. Spleen at five days after intravenous injection of $10^5LD_{50}$ of WE$_3$ strain LCM virus. Immunoglobulin deposits in necrotic zone in perifollicular region. Fluorescent antibody stain. X 600.
Figure 35. Spleen at six days after intravenous injection of $10^5 \text{LD}_{50}$ of WE$_3$ strain LCM virus. Severe follicular necrosis. Cuff of intact cells around central arteriole. Haematoxylin and eosin. X 200.
general increase in the number of pyroninophilic cells.

At four days, the pale cell areas around follicles were larger (Figure 31) and contained a few karyorrhectic nuclear fragments and pyroninophilic globules. Many of the large pale cells were pyroninophilic. Small amounts of an amorphous extracellular eosinophilic material were present in the pale cell areas. Pyroninophilic cells were common throughout the spleen.

By five days, spleen titres were maximal \((10^{6.4} \cdot LD_{50} \text{ per gram})\). The eosinophilic areas had become large acellular zones (Figure 32) and were extending into follicles, where the eosinophilic material was less plentiful. LCM virus antigen (Figure 33) and immunoglobulin deposits (Figure 34) were present especially at the edges of the eosinophilic areas, which contained a few intact cells, a few cells with vacuolated nuclei, and karyorrhectic nuclear fragments. The large pale cells had almost all disappeared.

At six days, the lesions were very much more severe and the "necrotic" zone had extended into the centre of many follicles (Figure 35). The follicles, consisting of nonstaining spaces in a faintly eosinophilic
Figure 36. Spleen at six days after intravenous injection of $10^5LD_{50}$ of WE$_3$ strain LCM virus. Severe necrosis. Haematoxylin and eosin. X 40.
matrix, contained erythrocytes and a few intact mononuclear cells, with a cuff of cells, most of them pyroninophilic, around the central arteriole. The spleens were small and pale and the sections were obviously pathological (Figure 36), even on naked eye examination.

The acellular eosinophilic material was faintly PAS positive, but all stains for amyloid were negative. Strain A mice are particularly susceptible to amyloid disease, (Dunn, 1944; West & Murphy, 1965), but the few perifollicular eosinophilic deposits which were produced by LCM virus in three-month-old strain A mice stained negatively for amyloid with Congo red and thioflavine T. The eosinophilic material also did not stain with pyronin or Gomori's silver stain, showing that it did not contain significant amounts of either RNA or reticulin. With Mallory's PTAH it stained reddish-brown, indicating that it contained no fibrin, and failure to stain with Van Gieson showed that there was no collagen. By histological criteria, therefore, the material is fibrinoid in type, since it is eosinophilic, PAS positive, stains reddish-brown with PTAH and does not stain with Van Gieson (Pearse, 1968).
Figure 37. Spleen at three days after intravenous injection of $10^5LD_{50}$ of WE$_3$ strain LCM virus. Large pale mononuclear cells in perifollicular region. Haematoxylin and eosin. X 600.
Both in the spleen and in lymph nodes (see page 79), the large pale cell areas which appeared at three days contained a prominent cell type (Figure 37). These cells were about twenty microns in diameter, with a pale-staining nucleus, a single large nucleolus and pyroninophilic cytoplasm. By five days, when the eosinophilic material was forming and the karyorrhectic nuclear remains were seen, these cells were less common, and they had disappeared by six days.

After a small intravenous injection of LCM virus \(10^2LD_{50}\) there were identical lesions in the spleen, but they appeared a day or so later. Less severe lesions were seen after footpad (subcutaneous) infection. In non-fatal infections, the spleen lesions resolved and normal appearances were restored within twenty to twenty-five days.

In summary, the eosinophilic spleen lesions in WEHI mice infected with LCM virus arise in perifollicular collections of large pale cells. Some of these cells are infected and some are pyroninophilic, presumably engaged in an immune response. As the lesion evolves there are signs of cell degeneration and the large pale cells disappear.
Factors Involved in the Production of Spleen Lesions in Mice Infected with LCM Virus

It was thought possible that the LCM virus stocks used to produce the lesions contained an additional pathogenic agent. This possibility was excluded by showing that plaque purified virus (Wainwright & Mims, 1967) still caused lesions and that the lesions were prevented by pre-incubating the inoculum with guinea pig antibody to LCM virus. It is also possible that WEHI mice carry an infectious agent that facilitates the production of lesions, which are much more severe in these mice than in any other strain tested. However, although spleen lesions are mild or minimal in C\textsubscript{57}BL, Bagg, C\textsubscript{3}H, CBA and A strain mice (see Section XI), they are of the same type as in the WEHI mice.

In groups of male and female C\textsubscript{57}BL mice, which had been caged for three months with WEHI mice of the same sex, infection with LCM virus still produced only mild spleen lesions (see Section XI), indicating that no infectious agent had been transmitted from the WEHI mice. However, when crosses were made between C\textsubscript{57}BL and WEHI mice, using as parents both males and females of each strain, the F\textsubscript{1} mice in all cases developed very
severe splenic lesions when infected with LCM virus (see Section XI). It appears, therefore, that there is a genetic basis for the susceptibility of WEHI mice to the development of splenic lesions in LCM virus infection.

The splenic lesions were not produced unless LCM virus grew to a significant extent in the spleen. Thus, the Armstrong strain and the carrier virus strain, which are not cleared from the blood of adult mice and show little or no growth in the spleen (see Section IV), did not produce lesions. Mere growth of virus in spleen and perifollicular cells, however, is not by itself enough. Mice from carrier colonies show extensive infection of the spleen (Mims, 1966a), as do mice infected neonatally, but splenic lesions are not seen. These mice do not have a significant immune response to infection, and it is this response which seems necessary if lesions are to appear. Even with the largest doses of virus given, spleen lesions were never seen until the fourth to fifth day, suggesting that they depend on the host immune response to infection. The presence of antibody in association with infected cells does not by itself appear to cause necrosis, because the injection of (heterologous) hyperimmune antiserum into infected mice did not precipitate the appearance of splenic lesions.
However, when mice were infected with WE3 strain LCM virus and given three injections of antilymphocyte serum on days 0, 1 and 2, splenic lesions did not occur. There is evidence that antilymphocyte serum does not prevent the production of antibody to LCM virus and does not inhibit the growth of the virus in tissues (Hirsch et al., 1967; 1968).

The experiment with antilymphocyte serum suggests that the cellular immune response, rather than antibody production, is necessary for the appearance of spleen lesions. For the following reasons it is thought that C57BL mice show a less vigorous cellular immune response to LCM virus infection than do WEHI mice. C57BL mice are highly resistant to the intracerebral lethality of LCM virus, although brain virus titres are as high as in WEHI mice (see Section XI). They show less footpad swelling with equal growth of virus in the foot after footpad infection and they die less readily, with less pleural fluid, after intraperitoneal infection (Section XI). Also, on recovering from infection they show a less marked delayed type hypersensitivity footpad response to LCM virus than do WEHI mice (Section XI). In a number of experiments, therefore, WEHI mice and mice of the C57BL strain were infected with a small footpad, intravenous or
intracerebral dose \(10^2 LD_{50}\) of \(WE_3\) strain LCM virus (see also Section XI). In the \(C_{57}\)BL mice, spleen lesions were very much less severe than in the WEHI mice and were sometimes absent. However, spleen titres at five days were higher than in WEHI mice, and neutralizing antibody titres at six days were the same (Mims, C.A., unpublished data). There were no differences in the pattern of infection of the spleens, as detected by fluorescent antibody staining. Thus, \(C_{57}\)BL mice showed far fewer and less severe spleen lesions than WEHI mice, in spite of greater growth of virus in the spleen and the same antibody response. Thus, the less severe spleen lesions in \(C_{57}\)BL mice are associated with a less intense cellular immune response to LCM virus infection.

In summary, the splenic lesions appear to be produced when there is virus growth in the spleen, probably in the perifollicular area, together with a local cellular immune response.

**Lymph Node Lesions in Mice Infected with LCM Virus**

In WEHI mice injected intravenously with \(10^5 LD_{50}\) of \(WE_3\) strain LCM virus, as in the above experiments, the first detectable changes in axillary and cervical lymph nodes were at three days, when small areas of large pale cells with occasional karyorrhectic nuclear debris were seen.
By four days, these areas had increased in extent and at five days, when nodes were enlarged, there was oedema and eosinophilic extracellular material originating in the large pale cell areas. Karyolytic nuclear remains were seen in the large pale cell areas, and there were scattered karyolytic nuclei and a reduction in the number of small lymphocytes throughout the node. At six and seven days mice were sick and dying and there was now a general karyorrhectic degeneration of lymphocyte nuclei.

To study changes in the local lymph node, WEHI mice were injected subcutaneously into the footpad with $10^5 \text{LD}_{50}$ of WE3 strain LCM virus and the popliteal lymph nodes from five mice were examined daily. By three days, the nodes were enlarged and there were foci of infection, up to fifteen cells in diameter, extending inwards from the marginal sinus. Pyroninophilic cells were common throughout the node and small areas of large pale cells had appeared. A few karyorrhectic nuclear fragments and some pyroninophilic globules were usually present in these areas. At four days, the nodes were oedematous and there were more pyroninophilic cells and by five days, extracellular eosinophilic material similar to that seen in spleens had appeared in the pale cell areas. Many nuclei
Figure 38. Popliteal lymph node at seven days after injection of $10^5LD_{50}$ of WE$_3$ strain LCM virus into the footpad. Advanced necrosis. Haematoxylin and eosin. X 40.
in these areas appeared vacuolated and karyolytic nuclear remains were present. At six days, there were extensive areas consisting of eosinophilic deposits and oedema, with scattered intact cells and nuclear debris. The edges of the eosinophilic deposits stained positively for LCM virus antigen and for mouse immunoglobulin. At seven days, all changes were more severe, many nodes containing no more than a few intact cells in a necrotic acellular reticulum (Figure 38).

On the eighth day, a small injection of India ink was made into the infected foot and the popliteal node was examined three hours later. Whereas in control mice the node was densely black, in infected mice the ink often filled the afferent lymphatics but usually failed to enter the swollen necrotic node. Similar observations were made on the twentieth day, when the popliteal node was smaller. After one year the popliteal node could not be detected when India ink was injected into the foot.

**Thymus Lesions in Mice Infected with LCM Virus**

In WEHI mice injected intravenously with a large dose \((10^5 LD_{50})\) of WE\(_3\) strain LCM virus, foci of infection were present in the thymus by three days. The foci originated from veins in the cortex and medulla. Histologically, however, there were no significant changes
Figure 39. Thymus at seven days after intravenous injection of $10^5LD_{50}$ of WE$_3$ strain LCM virus. Cortico-medullary inversion. Haematoxylin and eosin. X 40.
in the thymus until the fifth day, when there was depletion of lymphocytes in the cortex and a relative increase in the area occupied by the medulla. By six days, when mice were sick, the cortical cell depletion was more severe and thymuses were noticeably smaller. In the medulla, veins were dilated and karyorrhectic nuclear fragments were present inside reticular cells, among collections of small to medium lymphocytes. Where cell depletion in the cortex was less severe, similar evidence of lymphocyte destruction was present. At seven days, the thymuses were very small and the cortical cell depletion and increase in cell density in the medulla had led to inversion of the normal cortico-medullary appearances (Figure 39). In the medulla, reticular cells still contained karyorrhectic nuclear fragments. The eosinophilic lesions and the oedema produced in infected lymph nodes and spleen were not seen in the thymus.

Thymus changes were minimal or absent after footpad infection with LCM virus, when mice remained well. The decrease in thymus size, with cortical lymphocyte depletion and cell destruction, suggested that the changes were produced by the action of adrenal cortical hormones (Dougherty, 1952), presumably in response to the stress of
Figure 40. Thymus following repeated injection of hydrocortisone. Cortico-medullary inversion. Haematoxylin and eosin. X 40.
a severe infection. On the fifth day, the adrenal glands of the intravenously infected mice were pink rather than the normal yellow and at six days, when mice were sick, the glands were distinctly reddish. These changes suggest increased corticosteroid secretion, with accompanying lipid depletion and perhaps congestion of the gland. Similar thymus changes were produced when normal mice were injected subcutaneously with 1 mg hydrocortisone acetate (Roussel Laboratories Ltd., London) on days 0, 2 and 5, and thymuses examined histologically on day six (Figure 40). The changes were also seen when mice were sick, four to six days after a large intravenous dose of cowpox virus (Mims, 1968 a). In this infection there is no growth of virus in the thymus, but once again there was lipid depletion in adrenal glands. Bilaterally adrenalectomized mice failed to survive more than four days when infected with LCM virus, but in the case of cowpox virus the thymus changes were prevented in adrenalectomized mice (Wallnerova & Mims, 1969).

Thus, the thymus is infected by LCM virus, but changes in the thymus are probably caused by corticosteroids produced in increased amounts in response to the stress of a severe infection.
Figure 41. Liver at six days after intravenous injection of $10^5 \text{LD}_{50}$ of WE$_3$ strain LCM virus. Focus of parenchymal necrosis, associated with infiltrating mononuclear cells. Haematoxylin and eosin. X 400.
Figure 42. Liver at six days after intravenous injection of $10^5\text{LD}_{50}$ of WE3 strain LCM virus. Single necrotic hepatic cell and adjacent large pale mononuclear cell. Haematoxylin and eosin. X 600.
Figure 43. Liver at six days after intravenous injection of $10^5LD_{50}$ of WE$_3$ strain LCM virus. Mononuclear cells adhering to venous endothelium. Parenchymal necrosis. Haematoxylin and eosin. X 250.
Liver Lesions in Mice Infected with LCM Virus

In WEHI mice injected intravenously with $10^5 \text{LD}_{50}$ of WE$_3$ strain LCM virus, immunofluorescence observations showed foci of infection in the liver by two days, and at four days there were changes detectable by routine histology (see Section IV). These consisted of small focal infiltrates, mainly of mononuclear cells. By five and six days, the foci were larger and consisted of necrotic hepatic cells, eosinophilic material with interspersed nuclear remnants, erythrocytes and large pale mononuclear cells (Figure 41). Occasionally there was a single necrotic cell showing eosinophilic degeneration, with a large pale mononuclear cell attached to it (Figure 42). Some of the single necrotic cells were hepatic cells, but others were free in sinusoids and were presumably Kupffer cells or infiltrating cells. The eosinophilic material appeared similar to that seen in infected spleens and showed the same staining reactions with PAS, PTAH, Van Gieson and thioflavine T (see page 74). Also at five and six days, hepatic veins contained many large mononuclear cells (Figure 43) and there were periportal infiltrates. The infection was still focal, but a widespread fatty infiltration of hepatic cells was detectable by fettrot staining of formalin-fixed frozen
sections, confirming the observations of Lillie & Armstrong (1945). It was found that a similar fatty infiltration was produced when normal mice were injected subcutaneously with 1 mg hydrocortisone acetate (Roussel Laboratories Ltd.) on days 0, 2 and 5, and livers examined histologically on day six.

Although mice treated with three injections of antilymphocyte serum 0-2 days after infection showed no splenic necrosis (see page 78), the liver lesions still occurred, but were less severe. Since continuous treatment with antilymphocyte serum is necessary to prevent sickness in mice infected with LCM virus (Hirsch et al., 1967), it is possible that such treatment would have prevented liver lesions. As in the case of the splenic lesions, liver lesions did not occur in infections with the Armstrong strain of LCM virus or carrier virus (see also Section X), which do not grow to a significant extent in the liver (see Section IV).

Thus, the liver lesions occur when there is a significant degree of infection in the liver, accompanied by an infiltration of mononuclear cells.

**DISCUSSION**

Mice infected with LCM virus develop eosinophilic splenic lesions when the spleen is significantly infected.
and when at the same time there is a substantial immune response, probably local and cellular in nature. The lesions appear to originate in the perifollicular zone of large pale cells. If the LCM virus strain fails to infect the spleen, or if the cellular immune response is lacking, as in LCM carrier mice or following treatment with antilymphocyte serum, then lesions are not produced.

In the above studies, it was found that the eosinophilic lesions in the spleen contained degenerating large pale cells, LCM virus antigen and mouse immunoglobulin. There are great opportunities for error in the reconstruction of cellular events from a series of histological sections, but one possible interpretation of the LCM findings is as follows. Cells sensitized to LCM virus antigen are arising in the perifollicular areas and at the same time antigen is present either in the sensitized cells themselves, the infection being non-cytopathic, or in neighbouring cells. A local immunological interaction, similar to desensitization, then occurs between sensitized cells and antigen, and this results in cell destruction and also the deposition of eosinophilic material. In tissues like the spleen, containing dense local collections of sensitized cells together with antigen, cell damage would be severe. Perhaps when an individual
sensitized cell encounters an infected non-lymphoid cell there is an interaction which also results in cell damage, as postulated by Hotchin (1962a). With mumps virus infection in vitro, a cytocidal interaction has been demonstrated between sensitized lymphoid cells and infected cells (Speel et al., 1968), and suggestive evidence for a similar interaction has been obtained in the case of LCM virus infection in vitro (Lundstedt, 1969). The eosinophilic liver lesions may be interpreted on this basis, the infected hepatic or Kupffer cell undergoing eosinophilic degeneration after interaction with a sensitized cell. Antigen-antibody complexes, which are presumably formed in infected mice, could theoretically contribute to the liver lesions, as in "Auer" hepatitis (Paronetto and Popper, 1965). It is difficult to account for the widespread fatty infiltration in the liver in terms of the immune response, but increased corticosteroids produced during the stress of a severe infection may have produced this change. It was shown that a similar fatty infiltration was produced in the liver of normal mice by a series of injections of hydrocortisone.

The spleens of mice infected with LCM virus are very similar in appearance to the spleens of mice with
homologous disease (Congdon & Urso, 1957; Bradbury & Micklem, 1965), parabiosis intoxication (Cornelius et al., (2), 1968) and other graft-versus-host reactions (Cornelius, 1968; Cornelius et al., (1), 1968). In each instance, the spleen is the site of an immune interaction between lymphoid or lymphoreticular cells. In the case of LCM virus infection, the target cells could be host cells altered as a result of infection. Infected cells may be altered because cell membranes bear virus antigen, since LCM virus matures by budding from the cell surface (Dalton et al., 1968), or less probably because virus-induced transplantation antigens are present on infected cells. It has been found that skin grafts from LCM virus carrier mice are rejected by normal mice of the same strain (Holtermann & Majde, 1969).

In most of the graft-versus-host type reactions mentioned above, the perifollicular eosinophilic material has been shown to be amyloid. In some of these experiments, strain A mice, which are particularly susceptible to the development of amyloid (Dunn, 1944; West & Murphy, 1965), were used. However, even in strain A mice, the splenic lesions caused by LCM virus infection stained negatively for amyloid. Little is known about amyloid, which is produced under a wide variety of conditions,
usually involving immune responses. Muckle (1968) has pointed out that it is often produced when there is excess antigen in relation to immune reactivity. Perhaps amyloid production involves the presence of significant amounts of antigen, either cellular, particulate or soluble, in the near vicinity of collections of sensitized cells. This is the suggested mechanism for the production of eosinophilic material in mice infected with LCM virus.

Fibrinoid is another even more elusive eosinophilic material produced in conditions where cellular immune interactions are prominent. The eosinophilic material in mice infected with LCM virus is of fibrinoid type in that it is faintly PAS positive and stains reddish-brown with PTAH (Pearse, 1968). Willoughby & Spector (1964) have shown that disintegrated lymphoid cells may produce deposits of a fibrinoid-like material, a phenomenon that may well contribute to the production of the LCM lesions.

In mice injected intravenously with WE₃ strain LCM virus, changes in lymph nodes were considerable, though less severe than in the spleen. This would be consistent with less growth of virus in the lymph nodes than in the spleen and a less intense immune response. However,
in the local lymph node after footpad infection, with more intense immunological activity and probably more virus growth than in lymph nodes after systemic infection, the changes were much more severe and the eosinophilic deposits were more prominent. Deposits of a similar material were seen in the subcutaneous tissues of the footpad, after the primary footpad response and the hypersensitive footpad response to LCM virus (Section IX). The impaired lymph flow at eight days was not unexpected, the node by this time being reduced to a necrotic eosinophilic reticulum. There was no evidence for repair and reconstitution of the node, which was not detectable one year later. Hotchin (1962 b), in his original description of the primary footpad reaction of mice to LCM virus, referred to the "severe obliterative lesion" in the local lymph node.

Thymic lesions in mice infected with LCM virus were attributable to the action of corticosteroid hormones. In both spleen and lymph nodes, however, the immune response, corticosteroid hormones and eosinophilic lesions probably all contributed to the changes seen. The pale cell areas reflected the cellular immune response, the karyorrhesis and small lymphocyte depletion the corticosteroid action, and the eosinophilic deposits the LCM-type response. In most
studies of lymphoid tissue lesions in virus diseases
there have been no distinctions between pathogenetic
mechanisms of this sort and direct damage due to viral
growth in lymphoid cells. In LCM virus infection of
mice there is the unique possibility that infected lymphoid
cells can themselves take part in an immune response to the
infectious agent.

SUMMARY

Mice infected with LCM virus may show prominent
lesions in the spleen and their development has been
studied by immunofluorescence and histological examination.
The lesions were found to originate in perifollicular
collections of large pale cells and consisted of fibrinoid-
type deposits which often replaced lymphoid follicles.
They stained negatively for amyloid.

Similar lesions occurred in lymph nodes. In
local lymph nodes after footpad infection, the lesions were
very severe, leading to lymphatic blockade and
obliterative necrosis of the node.

The thymus showed changes attributable to the
action of corticosteroids. These changes are seen in
mice infected lethally with cowpox as well as in LCM virus
infection. They could be produced by injections of
hydrocortisone and prevented by bilateral adrenalectomy.
The pathogenesis of the fibrinoid-type lesions has been discussed, as well as the role of the immune response, of corticosteroids, and of direct viral activity in the pathogenesis of lymphoid lesions in LCM virus infection.

Mice infected with LCM virus developed focal eosinophilic necrosis in the liver and immunological mechanisms are thought to account for these lesions.

VII. THE ACTIVATION AND TOLERANCE OF MICE IMMUNOLOGICALLY TOLERANT TO LYMPHOCYTIC CHORDOMERGIC VIRUS

Mice infected with LCM virus developed focal eosinophilic necrosis in the liver and immunological mechanisms are thought to account for these lesions.
VII. THE ADOPTIVE IMMUNIZATION OF MICE IMMUNOLOGICALLY TOLERANT TO LYMPHOCYTIC CHORIOMENINGITIS VIRUS

When mature mice are infected with LCM virus, disease and death appear to be due to an immunological conflict in the host (Rowe, 1954; Hotchin, 1962a). In contrast, intrauterine or neonatal infection with LCM virus results in a lifelong virus carrier state in which the mice remain healthy despite the continual presence of virus in high titres in the blood and tissues (Traub, 1939; Hotchin & Cinite, 1958). Such mice are immunologically tolerant to LCM virus (Burnet & Fenner, 1949; Traub, 1960b).

Volkert (1965) and Volkert et al., (1964, 1965a) found that adoptive immunization of LCM virus carrier mice, with lymphoid cells from normal or immunized mice, resulted in the production of antibodies to the virus. However, there was no evidence of disease in the recipients, even when large numbers of immune lymphoid cells were transferred. Since reversal of immunological tolerance to LCM virus would be expected to cause disease, the effects of adoptive immunization of carrier mice have been re-investigated and Volkert's findings extended by immunofluorescence and histological studies.
RESULTS

Adoptive Immunization of LCM Virus Carrier Mice

Adult C_57BL and WEHI mice from LCM virus carrier colonies were injected intraperitoneally with cells taken from immune mice of the same strain. Adoptive immunization was also carried out on mice which had been neonatally infected with LCM virus. Each mouse was injected with $100 \times 10^6$ spleen and lymph node cells, together with peritoneal cells. Cells from peritoneal washings were included because of the probable importance of macrophages in immune responses (Fishman & Adler, 1963; Ford et al., 1966). Donor and recipient mice of the same sex were used, in order to avoid any histo-incompatibility arising from the transfer of male cells to a female recipient (Eichwald et al., 1957; Billingham & Silvers, 1960). Cells were also injected into normal, uninfected mice and into C_57BL carrier mice which had been irradiated twenty-four hours earlier.

Adoptive immunization produced no signs of illness in LCM virus carrier mice of either strain, in irradiated carrier mice or in normal mice.

Serum Antibody Titrations

Complement fixing antibody titres were determined in sera taken at intervals after adoptive
**Table 7.** COMPLEMENT FIXING ANTIBODY TITRES IN THE SERUM OF LCM VIRUS CARRIER MICE FOLLOWING ADOPTIVE IMMUNIZATION.

<table>
<thead>
<tr>
<th>LCM VIRUS CARRIER MICE</th>
<th>TIME AFTER ADOPTIVE IMMUNIZATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 week</td>
</tr>
<tr>
<td><strong>C57BL</strong></td>
<td>4/4*</td>
</tr>
<tr>
<td><strong>C57-BL IRRADIATED</strong></td>
<td>2/4</td>
</tr>
<tr>
<td><strong>WEHI</strong></td>
<td>4/4</td>
</tr>
</tbody>
</table>

* No. of sera positive/No. of sera tested

NT = Not tested
Figure 44. Complement fixing antibody titres in the serum of LCM virus carrier mice following adoptive immunization.
immunization. The results are shown in Table 7 and the median titres are charted in Figure 44. In adoptively immunized C_{57}BL carrier mice, maximal levels were reached by ten to fourteen days, with a decline by three to four weeks. It has been reported that, even in inbred mice, irradiation before the adoptive transfer of lymphoid cells results in the production of higher levels of antibodies (Mitchison, 1957; Makela & Mitchison, 1965; Celada, 1966). When C_{57}BL carrier mice were irradiated before adoptive immunization, antibody titres did reach higher levels and were maintained for a much longer period (Table 7; Figure 44), antibodies still being present twelve weeks after cell transfer. In WEHI carrier mice, in contrast, antibody titres were much lower and in most mice were not detectable by two weeks after adoptive immunization.

Tests for neutralizing antibody were performed on the sera of five C_{57}BL carrier mice six weeks after adoptive immunization and four of the five sera were positive. The median value of the Neutralization Indices was 60.

Antibodies were never detected in the blood of either normal carrier mice or those which had received irradiation alone. Transfer of immune lymphoid cells to normal mice did not result in detectable antibody formation.
Infectivity Titrations

The blood virus titres of nine normal C₅₇BL carrier mice were each greater than 10⁴.⁰ LD₅₀ per ml. In six C₅₇BL carrier mice which had received irradiation only, blood virus titres, tested during a period of six months, were always greater than 10⁴.⁵ LD₅₀ per ml. In contrast, eight out of nine adoptively immunized C₅₇BL carrier mice, tested up to six weeks after cell transfer, had blood virus titres of less than 10³.⁰ LD₅₀ per ml.

The spleens of normal C₅₇BL carrier mice, and of those which had received irradiation only, showed a marked variability in the amounts of virus present. However, four of eight tested had titres greater than 10².⁵ LD₅₀ per gram. Eight adoptively immunized carriers, tested up to six weeks after cell transfer, all had spleen titres less than 10².⁵ LD₅₀ per gram.

Immunofluorescence

The tissues of adoptively immunized LCM virus carrier mice, irradiated or non-irradiated, were compared with those of appropriate normal carrier mice. The tissues of more than fifty adoptively immunized carrier mice were examined by immunofluorescence at periods varying from one week to twelve weeks after cell transfer. They showed no detectable difference from control carrier
mice in the distribution of LCM virus antigen. The microscopic appearances were identical with those described for normal LCM virus carrier mice by Wilsnack & Rowe (1964) and Mims (1966a). Whether or not they had been adoptively immunized, no differences were detected between irradiated and non-irradiated carrier mice or between mice of a carrier colony and neonatally infected mice. It may be noted that infected white blood cells and platelets were still present in blood smears of the carrier mice after adoptive immunization.

Staining of immunoglobulin in the tissues of adoptively immunized carrier mice showed no detectable differences from those of normal carrier mice, with the exception of the kidneys. Some glomeruli contained greater immunoglobulin deposits than were present in any normal carrier mice of the same age; the appearance was in fact similar to that seen in old carrier mice. Further investigations of glomerular immunoglobulin deposits are reported on page 99.

Histology

Histological examination of haematoxylin and eosin stained tissues of adoptively immunized carrier mice showed no significant differences from the appearances seen in normal carrier mice. Normal LCM virus carrier mice
showed a tendency to mild mononuclear cell infiltrations in the meninges, liver, lung, kidney, pancreas and intestine, and some of these cells were pyroninophilic. Methyl green-pyronin staining of the spleen and lymph nodes of carrier mice one week after adoptive immunization showed larger numbers of pyroninophilic cells than in normal carrier mice.

Thus, the transfer of immune lymphoid cells to LCM virus carrier mice resulted in the production of antibodies, but no evidence of a cellular hypersensitivity reaction was seen.

Further Experiments with Adoptive Immunization

The possibility was considered that sensitization of the donor lymphoid cells may have diminished by the time they were taken for transfer, several weeks after immunization. One indication of cellular hypersensitivity to LCM virus is the development of footpad swelling twenty-four hours after a challenge injection of virus into the footpad (see Section IX). This hypersensitivity response is maximal at about ten days after a primary infection, suggesting that maximal sensitization of lymphoid cells occurs at this time. In some experiments, therefore, cells for adoptive immunization were obtained from mice six days and ten days after primary infection with LCM virus.
However, the results were no different than with cells from hyperimmunized donors.

In delayed type hypersensitivity reactions to tuberculin, Lubaroff & Waksman (1968 a, b) have produced evidence that transferred lymphoid cells exert their hypersensitivity response in association with bone marrow cells of the recipient. It was thought possible that in LCM virus carrier mice, the bone marrow cells may be unable to take part in a hypersensitivity response, whereas the transferred cells could themselves produce an antibody response. Experiments were therefore carried out in which approximately $10^6$ immune bone marrow cells from the donor mice were added to the spleen, lymph node and peritoneal cells used for adoptive immunization (see Section II). However, the effects were no different than in lymphoid cell transfer without the inclusion of bone marrow cells.

Deposition of Immunoglobulin in the Kidneys of LCM Virus Carrier Mice

Immunoglobulin was present in the glomeruli of adoptively immunized carrier mice in greater amounts than in normal carrier mice of the same age (see page 97) and this was investigated in more detail. Frozen sections of the kidneys of normal and LCM virus carrier mice, of both C57BL and WEHI strains, were stained with fluorescein-
<table>
<thead>
<tr>
<th>AGE</th>
<th>NORMAL MICE</th>
<th>CARRIER COLONY MICE</th>
<th>NEONATALLY INFECTED MICE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proportion of glomeruli fluorescent</td>
<td>Degree of fluorescence</td>
<td>Proportion of glomeruli fluorescent</td>
</tr>
<tr>
<td>3 days</td>
<td>2% +</td>
<td>20% ++</td>
<td>ND</td>
</tr>
<tr>
<td>4 weeks</td>
<td>5% +</td>
<td>100% +++</td>
<td>ND</td>
</tr>
<tr>
<td>3 months</td>
<td>ND ND</td>
<td>100% +++++</td>
<td>100% ++++</td>
</tr>
<tr>
<td>6 months</td>
<td>ND ND</td>
<td>100% +++++</td>
<td>ND</td>
</tr>
<tr>
<td>18 months</td>
<td>30% +</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Graded according to amount and brightness of fluorescence in glomeruli.

ND = Not done

**Table 8.** DEPOSITION OF IMMUNOGLOBULIN IN THE GLOMERULI OF C₅₇BL AND WEHI MICE.
**Figure 45.** Kidney of three-day-old mouse from an LCM virus carrier colony. Immunoglobulin deposits in glomerulus. Fluorescent antibody stain. X 600.
Figure 46. Kidney of three-month-old mouse from an LCM virus carrier colony. Immunoglobulin deposits in glomeruli. Fluorescent antibody stain. X 600.
Figure 47. Kidney of three-day-old mouse from an LCM virus carrier colony. LCM virus antigen in glomeruli. Fluorescent antibody stain. X 300.
conjugated goat antiserum globulin against mouse serum IgG globulin (Microbiological Associates). Kidneys from groups of at least six mice were examined and the results are shown in Table 8. No significant differences were found between mice of the two strains. Small amounts of immunoglobulin were present in a few glomeruli in young normal mice and in a greater number of glomeruli in old normal mice. In contrast, LCM virus carrier mice showed larger amounts of immunoglobulin in twenty per cent of glomeruli, even at three days of age (Figure 45), and the immunoglobulin deposits increased in amount and frequency in older carrier mice (Figure 46). The glomeruli also stained for LCM virus antigen (Figure 47). The kidneys of neonatally infected mice, examined when they were three months old, showed similar glomerular immunoglobulin deposits to those seen in LCM virus carrier colony mice of the same age.

The following tests were done to check the specificity of staining for mouse immunoglobulin. Fluorescein-conjugated goat antiserum globulin against the IgG globulin of guinea pig or chicken serum (Microbiological Associates) gave no glomerular staining. Pretreatment with the 7S fraction of goat antiserum against mouse serum IgG globulin (Cappel Laboratories, Downingtown, Pasadena) resulted in marked quenching of fluorescent staining of the
glomeruli by the anti-mouse conjugate, whereas pre-treatment with the 7S fraction of goat antiserum against guinea pig serum IgG globulin (Cappel Laboratories) did not. Evidence for the Presence of Circulating Infectious Virus-Antibody Complexes in LCM Virus Carrier Mice

If the immunoglobulin demonstrated in the glomeruli of carrier mice represents antibody to LCM virus, its possible origin would be from the deposition of circulating virus-antibody complexes, a phenomenon which is known to occur experimentally (Dixon et al., 1961; Pincus et al., 1968). Infectious virus-antibody complexes have been demonstrated in the serum of mice infected with lactic dehydrogenase virus (Notkins et al., 1966, 1968), and of mink infected with the virus of Aleutian disease (Porter & Larsen, 1967), both infections causing a lifelong viraemia. The following experiment was therefore performed with LCM virus carrier mice. Serum from C\textsubscript{57}BL carrier mice was centrifuged at 2,500g to deposit platelets. It was then mixed with an equal volume of goat antiserum globulin against mouse serum IgG globulin (Microbiological Associates) and complement added. As controls, carrier serum was mixed with goat antiserum globulin against chicken serum IgG globulin (Microbiological Associates) plus complement, and with gelatin saline. The mixtures were incubated for one hour at 37\textdegree C., centrifuged at 2,500 g for twenty
Table 9. EFFECT OF INCUBATING SERUM FROM LCM VIRUS CARRIER MICE WITH ANTISERUM AGAINST MOUSE IMMUNOGLOBULIN.

<table>
<thead>
<tr>
<th>SERUM FROM LCM VIRUS CARRIER MICE*</th>
<th>ANTISERUM</th>
<th>GELATIN SALINE</th>
<th>VIRUS TITRE** (log₁₀LD₅₀ per ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>Anti-Mouse Immunoglobulin</td>
<td>-</td>
<td>2.5</td>
</tr>
<tr>
<td>+</td>
<td>Anti-Chicken Immunoglobulin</td>
<td>-</td>
<td>4.2</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>3.8</td>
</tr>
</tbody>
</table>

* Complement added

** Reaction mixture incubated for 1 hour at 37°C, centrifuged and supernate titrated.
minutes and the supernates titrated intracerebrally in mice. The results are shown in Table 9. It can be seen that the titre of the test reaction mixture of carrier serum with anti-mouse immunoglobulin was reduced by more than ninety per cent, in comparison with the two control reaction mixtures. This experiment indicates that antiserum to mouse immunoglobulin caused a marked reduction in the virus titre in carrier serum by combining with and precipitating out mouse immunoglobulin which was combined with virus yet did not neutralize virus infectivity.

To check that LCM virus was not adsorbed onto mouse antigen-antibody complexes in a non-specific fashion, the following test was done. Normal mouse serum was mixed with LCM virus, antiserum against mouse immunoglobulin and complement. Normal mouse serum was also mixed with virus, antiserum against chicken immunoglobulin and complement. After incubation and centrifugation as described previously, the supernates were titrated intracerebrally in mice. No reduction in virus titre occurred, showing that there was no significant non-specific adsorption of virus to immune complexes.

DISCUSSION

Adoptive immunization of LCM virus carrier mice
resulted in the formation of complement fixing (CF) and neutralizing antibodies, confirming the findings of Volkert et al., (1964). There is evidence that in recipients of lymphoid cells transferred from an immunized donor, antibody is produced by the transferred cells themselves (Grey, 1962).

Peak antibody titres have been found to occur in mice at about two weeks after the adoptive transfer of immune lymphoid cells followed by a single antigenic challenge (Makela & Mitchison, 1965; Celada, 1966). After the adoptive immunization of C57BL LCM virus carrier mice, in which viral antigen is continually present, the CF antibody response reached a maximum at about two weeks (Figure 44). The fall in CF antibody levels in C57BL carrier mice by three to four weeks after adoptive immunization suggests that the transferred cells were rejected by the recipient. This may seem unexpected, in view of the fact that the C57BL mice used were inbred, though not strictly brother-sister mated, and a small number of mice tested appeared to accept skin grafts from each other. It is possible, however, that some histo-incompatibility may have arisen in the C57BL colony. Wakefield & Rose (1968) have shown that rejection of transferred lymphoid cells is a more sensitive indication of minor degrees of tissue incompatibility than rejection of skin grafts. Even parental
C$_{57}$BL lymphoid cells transplanted to theoretically tolerant (C$_{57}$BL X A)$_1$F$_1$ hybrid recipients survived only about two weeks, as indicated by their ability to produce antibody (Boyse, 1959). In the C$_{57}$BL mice, the presence of minor histo-incompatibilities is suggested by the increased duration of CF antibody production when the recipients of cell transfer had been irradiated (Figure 44). In the adoptive immunization experiments of Volkert et al. (1964), in which "highly inbred" mice were used, CF antibodies to LCM virus were still detectable fifteen months after cell transfer. In the adoptively immunized WEHI carrier mice, the CF antibody titres had fallen to undetectable levels by two weeks (Figure 44). This would be expected, because WEHI mice are not inbred and the transferred cells would have been rejected at an early stage.

It may be noted that irradiation of LCM virus carrier mice did not by itself result in antibody production or depression of blood virus titres. Sublethal irradiation is known to cause the breakdown of partial tolerance to a protein antigen (Makela & Nossal, 1962), but does not terminate a more complete tolerance (Weigle, 1964). The fact that blood from adoptively immunized LCM virus carrier mice showed a marked reduction in virus titre is to be expected, since free virus would be neutralized by
antibodies in the serum. Similarly, the decreased virus titres found by Volkert (1963) in the tissue suspensions of adoptively immunized LCM virus carrier mice could have been due to the presence of neutralizing antibodies in the blood contained in the tissues. The injection of immune serum was found to cause a temporary depression of blood virus titres (Volkert & Hannover Larsen, 1965 b). However, circulating antibodies would not be expected to affect the production of virus within infected cells and in fact, immunofluorescence staining of LCM virus antigen in the tissues after adoptive immunization showed no significant differences from that seen in normal carrier mice. Infection also persisted in circulating white blood cells and platelets.

There is a marked reduction in severity of the disease caused by LCM virus when animals are infected following X-irradiation (Rowe, 1956; Hotchin & Weigand, 1961 b; Collins et al., 1961), neonatal thymectomy (Rowe et al., 1963; Levey et al., 1963; East et al., 1964; Hotchin & Sikora, 1964) or treatment with antimetabolites (Haas et al., 1956, 1957; Lerner & Haas, 1958; Levy & Haas, 1958), cortisone (Hotchin & Cinitis, 1958) or antilymphocyte serum (Gledhill, 1967; Hirsch et al., 1967). Accumulated evidence from these and other experiments has led to the association of the cellular immune response with the development of disease and death in animals infected with
LCM virus (Hotchin, 1962 a). However, despite the presence of large numbers of immune lymphoid cells in the adoptively immunized LCM virus carrier mice, they remained quite healthy. Histological examination of the tissues showed only the mild mononuclear cell infiltrations seen in normal carrier mice. The absence of sickness or death in LCM virus carrier mice after the transfer of immune lymphoid cells has also been noted by Volkert et al., (1964) and Hotchin (1962 a, 1965), but no histological or immunofluorescence studies were reported by these authors. This could simply be because, in comparison with the enormous numbers and wide distribution of infected cells in the carrier mice, there are insufficient transferred cells to produce histologically visible lesions. However, Volkert et al., (1964) have transferred up to $2,000 \times 10^6$ immune cells without obvious ill effects, and it might be expected that multiplication of the transferred cells would occur, on antigenic stimulation.

By adoptive immunization, therefore, the immunological tolerance of the LCM virus carrier mice has been reversed in so far as humoral antibody production is concerned. The mice remained healthy, indicating that antibody to LCM virus is not by itself responsible for sickness or pathological lesions. Similarly, in the
experiments of Volkert et al., (1964), adoptively immunized LCM virus carrier mice did not become sick despite the production of very high levels of antibody. This suggests that the adoptively immunized mice were still incapable of a cellular hypersensitivity response to LCM virus. There are several analogous situations in which there may be a dissociation between cellular and humoral immune responses.

Desensitization refers to the ability of antigen to depress the expression of delayed type hypersensitivity (DTH) in animals which already manifest DTH. Even microgram amounts of antigen may be sufficient to decrease the DTH response (Uhr & Pappenheimer, 1958), and antigen probably acts directly on the cells which mediate DTH (Asherson & Stone, 1967). In LCM virus carrier mice, the transferred sensitized cells immediately come into contact with antigen and their ability to manifest a hypersensitivity reaction could thereby be depressed. Antigen-mediated depression of immune responses may also selectively affect the induction of DTH, while leaving the circulating antibody response relatively intact; this has been termed "immune deviation" or "split tolerance" (Asherson & Stone, 1965; Crowle & Hu, 1966; Borel et al., 1966). It seems to be an effect on the lymphoid cells themselves, because
lymphoid cells from "deviated" animals show a reduced ability to transfer DTH to normal recipients (Asherson, 1966). In certain experimental situations, there is evidence for the presence of split tolerance to LCM virus antigens (Hirsch et al., 1968; Hannover Larsen, 1969). The adoptive immunization of LCM virus carrier mice may result in a condition resembling split tolerance, with the transferred lymphoid cells capable of producing antibodies, but incapable of initiating a cellular hypersensitivity reaction which would result in disease and death. It has also been found that antiserum to certain antigens, injected during the early period of sensitization, causes partial suppression of DTH (Crowle & Hu, 1965; Axelrad, 1968). More directly comparable to the results of adoptive immunization of LCM virus carrier mice is the following finding with experimental allergic encephalomyelitis (EAE), which is considered to be caused by a cellular hypersensitivity reaction (David & Paterson, 1965; Falk et al., 1968). Although EAE can be transferred to normal animals by means of sensitized lymphoid cells, injection of the protein antigen into the recipients at about the time of cell transfer prevents the development of EAE (Levine & Hoenig, 1968).
All these examples show that DTH can be suppressed, in various circumstances, by both antigen and antibody. When immune lymphoid cells are transferred to LCM virus carrier mice, various factors operate—the immediate contact with antigen, the presence of sub-threshold amounts of antibodies and perhaps antigen-antibody complexes, and the immediate stimulation of the transferred cells to produce antibodies themselves. The most important factor in suppressing the manifestation of a cellular hypersensitivity reaction is probably the immediate contact with antigen, which is widespread in LCM virus carrier mice, resulting in desensitization of the transferred cells. The other factors may play subsidiary roles. There remains the possibility that in LCM virus infection of mice, the humoral antibody response may be directed against one antigen or antigenic determinant and the cellular hypersensitivity response directed against another, perhaps even against an altered host cell antigen. The two types of immune response may therefore be independent and reversal of tolerance to one may not necessarily affect the other.

It has long been thought that LCM virus carrier mice are incapable of either an antibody response or a cellular hypersensitivity response to the virus. However,
the detection of immunoglobulin in the kidneys of mice of LCM virus carrier colonies suggests that tolerance is subtotal. The same was found in mice which had been neonatally infected with LCM virus, confirming the observations of Oldstone & Dixon (1967), who eluted the immunoglobulin from the kidneys and showed that it would fix complement in the presence of LCM virus antigen.

Low levels of antibody to LCM virus have also been detected in the plasma of neonatally infected mice (Benson & Hotchin, 1969). It therefore appears that, in spite of the fact that they may have been infected from as early a stage as the ovum (Mims, 1966 a), the mice of an LCM virus carrier colony do produce small amounts of antibody to LCM virus. Evidence has been obtained for the presence of circulating infectious virus-antibody complexes, and antibody to LCM virus probably accumulates in the glomeruli of carrier mice by the deposition of these complexes.

A disease of late onset occurs in certain strains of mice between two and eighteen months after neonatal LCM virus infection (Hotchin & Collins, 1964; Baker & Hotchin, 1967; Oldstone & Dixon, 1969). An important finding in these mice is a chronic glomerulonephritis, which appears to result from the deposition of circulating virus-antibody complexes in the glomeruli (Oldstone & Dixon, 1969).
Deposition of antigen-antibody complexes in the glomeruli has been shown to cause glomerulonephritis in serum sickness following the injection of foreign serum proteins (Dixon et al., 1958; McCluskey & Benacerraf, 1959), and in NZB mice making antibodies to their own DNA (Lambert & Dixon, 1968). Following treatment with anti-thymocyte serum, infant mice infected with LCM virus produced both circulating antibody and virus for a prolonged period, and they developed glomerulonephritis at an early age (Hirsch et al., 1968 (1), (2)). The observed increase in glomerular immunoglobulin deposits in LCM virus carrier mice after adoptive immunization was probably due to the presence of higher levels of circulating virus-antibody complexes than in normal carrier mice of the same age. The adoptively immunized mice were stimulated to produce large amounts of antibodies, which could have formed complexes with the virus which was continually being released into the blood.

An interesting finding was that normal mice of the C57BL and WEHI strains showed small amounts of immunoglobulin in some glomeruli. It is possible that these mice were carriers of an agent other than LCM virus, perhaps a murine leukaemia virus. If, in spite of intrauterine infection, small amounts of antibody were also present in the glomeruli of normal, uninfected mice.
produced, then circulating virus-antibody complexes could have localized in the renal glomeruli, as in LCM virus carrier mice. In mice carrying Moloney leukaemia virus, there is evidence that virus-antibody complexes are deposited in the renal glomeruli (Hirsch et al., 1969).

**SUMMARY**

The adoptive transfer of immune lymphoid cells to mice immunologically tolerant to LCM virus resulted in the production of humoral antibodies. Circulating free virus was neutralized, but intracellular production of virus was unaffected, as detected by immunofluorescence staining of antigen. There was no clinical or histological evidence of disease, suggesting that the adoptively immunized carrier mice were still incapable of a cellular hypersensitivity response against the virus. Possible explanations for this dissociation of cellular and humoral immune responses have been discussed.

Evidence has been presented for the production of small amounts of antibody by LCM virus carrier mice and the presence of infectious virus-antibody complexes in their serum. Immunoglobulin deposits were detected in the glomeruli of infant carrier mice and increased with advancing age. Smaller immunoglobulin deposits were also present in the glomeruli of normal, uninfected mice.
THE EFFECTS OF BORDETELLA PERTUSSIS VACCINE AND FREUND'S ADJUVANT ON THE RESPONSE OF MICE TO INFECTION WITH LYMPHOCYTIC CHORIOMENINGITIS VIRUS.

The injection of an animal with Bordetella pertussis vaccine induces many biological changes, the mechanisms of which have not been clearly defined (Pittman, 1957; Kind, 1958; Munoz, 1964; Levine & Pieroni, 1966). Pertussis vaccine acts as an adjuvant in increasing antibody production (Kind, 1957; Farthing, 1961; Munoz, 1963), induces a marked lymphocytosis (Tuta, 1937; Morse, 1965), and increases sensitivity to histamine, serotonin and anaphylaxis (Parfentjev & Goodline, 1948; Malkiel & Hargis, 1952; Munoz, 1957). In various experimental circumstances, pertussis vaccine has been found to confer either increased resistance or increased susceptibility to infection with certain micro-organisms (Parfentjev, 1953, 1955; Dubos & Schaedler, 1956; Arch & Parfentjev, 1957; Abrahams, 1966). In addition, it has been shown to enhance the production of experimental auto-immune diseases, such as allergic encephalomyelitis (Lee & Olitsky, 1955), adrenalitis (Levine & Wenk, 1968) and thyroiditis (Paterson & Drobish, 1968), possibly by causing an increased cellular hypersensitivity response, by an effect on the lymphoid system (Levine & Wenk,
1967). There is also evidence that in certain circumstances pertussis vaccine causes some depression of the cellular hypersensitivity response. For example, it may depress the tuberculin reaction in the skin (Floersheim, 1965) and enable increased growth of transplantable tumours (Hirano et al., 1967; Floersheim, 1967).

Freund's complete adjuvant is known to promote both antibody production and the induction of delayed type hypersensitivity responses (Freund, 1956). Oily adjuvants containing mycobacteria have a profound effect on the lymphoreticular system, as illustrated by their induction of disseminated granulomatous lesions in certain species (Rist, 1938; Chase, 1959; Laufer et al., 1959; Steiner et al., 1960).

The cellular immune response plays an important part in disease and death caused by LCM virus infection (Hotchin, 1962 a). Since both pertussis vaccine and Freund's adjuvant have a profound effect on the immune response, in particular on the cells of the lymphoreticular system, their effect on the pathogenesis of LCM virus infection in mice has been investigated.
<table>
<thead>
<tr>
<th>STRAIN OF MICE</th>
<th>STRAIN OF VIRUS</th>
<th>ROUTE OF INJECTION</th>
<th>LCM VIRUS (10^2 LD_{50} on Day 0)</th>
<th>PERTUSSIS VACCINE (10^{10} organisms intraperitoneally on Day 3)</th>
<th>NUMBER OF MICE TESTED</th>
<th>RESULT</th>
</tr>
</thead>
<tbody>
<tr>
<td>WEHI</td>
<td>WE_3</td>
<td>INTRACEREBRAL</td>
<td>-</td>
<td>+</td>
<td>36</td>
<td>100% DEATHS AT 7-8 DAYS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>INTRAPERITONEAL</td>
<td>-</td>
<td>+</td>
<td>66</td>
<td>20% DEATHS AT 8-12 DAYS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FOOTPAD (Subcutaneous)</td>
<td>-</td>
<td>+</td>
<td>36</td>
<td>FOOTPAD SWELLING AT 7 DAYS NO SICKNESS</td>
</tr>
<tr>
<td>ARM-STRONG</td>
<td></td>
<td>INTRACEREBRAL</td>
<td>-</td>
<td>+</td>
<td>12</td>
<td>100% DEATHS AT 6-8 DAYS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>INTRAPERITONEAL</td>
<td>-</td>
<td>+</td>
<td>12</td>
<td>NO SICKNESS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FOOTPAD (Subcutaneous)</td>
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<td>+</td>
<td>12</td>
<td>FOOTPAD SWELLING AT 8 DAYS NO SICKNESS</td>
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<td></td>
<td></td>
<td></td>
<td>-</td>
<td>+</td>
<td>12</td>
<td>NO SICKNESS</td>
</tr>
<tr>
<td></td>
<td>C_57BL</td>
<td>WE_3</td>
<td>INTRAPERITONEAL</td>
<td>-</td>
<td>18</td>
<td>50% MILD SICKNESS AT 10-12 DAYS. NO DEATHS</td>
</tr>
<tr>
<td>WEHI CARRIER MICE</td>
<td>WE_3</td>
<td>INTRAPERITONEAL</td>
<td>-</td>
<td>+</td>
<td>12</td>
<td>NO SICKNESS</td>
</tr>
</tbody>
</table>

Table 10. EFFECTS OF PERTUSSIS VACCINE ON THE RESPONSE OF MICE TO LCM VIRUS INFECTION.
RESULTS

A. Effects of Pertussis Vaccine on the Response of Mice to LCM Virus Infection

Mice were infected with a standard dose of $10^2LD_{50}$ of LCM virus. *Bordetella pertussis* vaccine (Commonwealth Serum Laboratories, Melbourne) was injected intraperitoneally in a dose of $0.25 \text{ ml containing } 10^{10}$ killed organisms, four days before, one day after or three days after LCM virus infection. Similar effects were noted with all these schedules, but were most marked when the pertussis vaccine was injected three days after infection. Results from groups of mice receiving pertussis vaccine on day three are shown in Table 10.

Intracerebral Infection

In WEHI mice infected intracerebrally with either the WE$_3$ or the Armstrong strain of LCM virus, pertussis vaccine caused delayed mortality. As is usual after intracerebral infection with LCM virus, the mice died in convulsions with hind limbs extended, and tail spinning of sick mice caused convulsions. An experiment was done to check that pertussis vaccine did not cause decreased or delayed growth of LCM virus in the brain, and to investigate its effect on growth of
ROUTE OF INFECTION WITH LCM VIRUS (WE₃ strain 10⁻² LD₅₀ on day 0)

<table>
<thead>
<tr>
<th>ROUTE OF INFECTION</th>
<th>PERTUSSIS VACCINE (0.25 ml intra-peritoneally on day 3)</th>
<th>VIRUS TITRES (log₁₀ LD₅₀ per gram)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BRAIN</td>
</tr>
<tr>
<td>INTRACEREBRAL</td>
<td>-</td>
<td>Day 5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>6.8</td>
</tr>
<tr>
<td>INTRA-PERITONEAL</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>FOOTPAD</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>(Subcutaneous)</td>
<td>+</td>
<td></td>
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</tbody>
</table>

**Table 11.** EFFECT OF PERTUSSIS VACCINE ON GROWTH OF VIRUS IN THE BRAIN AND LIVER OF WEHI MICE INFECTED WITH LCM VIRUS.
virus in the viscera after intracerebral infection. Twelve mice were injected intracerebrally with WE₃ strain virus and half received pertussis vaccine intraperitoneally at three days. At five days, pooled brains and livers from each group were titrated and the results are shown in Table 11. In the brain, virus had grown to identical titres whether the mice had received pertussis vaccine or not, but virus had grown to higher titres in the livers of mice which had received pertussis vaccine.

**Intraperitoneal Infection**

After intraperitoneal infection with the WE₃ strain of LCM virus, pertussis vaccine caused earlier and increased mortality in WEHI mice (Table 10). To investigate whether pertussis vaccine affected the growth of virus in the viscera, the following experiment was done. Twelve mice were infected intraperitoneally with WE₃ strain LCM virus and at three days, half received pertussis vaccine intraperitoneally. At six days, pooled livers from each group were titrated and the results are shown in Table 11. It can be seen that the virus had grown to higher titres in the livers of mice which had received pertussis vaccine. The Armstrong strain of LCM virus grows to a significant extent in mice only after injection directly into the brain (Lehmann-Grube, 1964 a; Section X).
and intraperitoneal infection with this strain did not cause illness and was not affected by pertussis vaccine (Table 10). C₅₋BL mice are much less susceptible than WEHI mice to the lethal effects of LCM virus, although it grows to a similar extent in their tissues (see Section XI). C₅₋BL mice did not die after intraperitoneal infection with the WE₃ strain virus alone, but the injection of pertussis vaccine resulted in fifty per cent mortality (Table 10). In WEHI strain LCM virus carrier mice, which are immunologically tolerant to the virus, intraperitoneal injection of WE₃ strain LCM virus and pertussis vaccine did not cause illness (Table 10).

Footpad Infection

After subcutaneous injection of LCM virus into the footpad, swelling occurred at seven or eight days, depending on the strain of virus, but no sickness or deaths occurred (Table 10; see also Section X). However, when the mice received pertussis vaccine, footpad swelling was delayed for one day (Table 10). Some of the mice infected with WE₃ strain virus died, having shown no footpad swelling; most of the remainder became sick and recovered, and these developed less footpad swelling than mice that remained healthy. The following experiment was done to investigate whether pertussis vaccine affected the
growth of virus in the viscera, after footpad injection. Twelve mice were infected subcutaneously in the footpad with LCM virus and at three days, half were injected intraperitoneally with pertussis vaccine. At seven days, pooled livers from each group were titrated and the results are shown in Table 11. It can be seen that the virus grew to higher titres in the livers of mice which had received pertussis vaccine.

Thus, pertussis vaccine delayed death in mice infected intracerebrally with LCM virus, but caused earlier and increased mortality in intraperitoneally infected mice and caused death in fifty percent of footpad infected mice.

Effect of Pertussis Vaccine on Antibody Production

Kind (1957) showed that when pertussis vaccine was injected intraperitoneally at the same time as red blood cells, increased haemagglutinin production occurred. Since in the above experiments pertussis vaccine was injected three days after LCM virus infection, the effect of pertussis given three days after red blood cells was tested. Twenty mice were injected intraperitoneally with $3 \times 10^6$ sheep red blood cells in normal saline and at three days, half received an injection of pertussis vaccine intraperitoneally. Serum haemagglutinating antibody
titres were tested at seven days and showed a median titre of 1:640, whether the mice had received pertussis vaccine or not. That is, the injection of pertussis vaccine three days after the antigen did not influence antibody titres by seven days.

Effect of Pertussis Vaccine on the Cellular Hypersensitivity Response

In the above experiments, pertussis-treated mice showed a delay in footpad swelling after local injection of LCM virus. The pertussis vaccine was injected about four days before the usual onset of footpad swelling. It has been shown that the immune response to LCM virus infection plays a large part in primary footpad swelling, and in hypersensitive footpad swelling the effect of the cellular immune response predominates (see Section IX). Since pertussis vaccine did not appear to act as an adjuvant in increasing humoral antibody production to sheep red blood cells when it was injected three days after the antigen, its effect on the expression of hypersensitive footpad swelling was investigated. Thirty mice were injected intraperitoneally with $10^4 LD_{50}$ of Armstrong strain LCM virus and at seven days, half received an intraperitoneal injection of pertussis vaccine. At ten days, both groups of mice were injected in the
<table>
<thead>
<tr>
<th>VIRUS</th>
<th>PERTUSSIS VACCINE (10^10 organisms intraperitoneally 3 days before virus injection)</th>
<th>MEAN PERCENTAGE INCREASE IN FOOTPAD THICKNESS (Time after injection of virus eliciting hypersensitive footpad swelling)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4 HOURS</td>
</tr>
<tr>
<td>LCM (10^7 LD_50</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>subcutaneously into footpad)</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>COWPOX (10^7 pfu</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>subcutaneously into footpad)</td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

* Standard Deviation
ND = Not done

**Table 12.** EFFECT OF PERTUSSIS VACCINE ON HYPERSENSITIVE FOOTPAD SWELLING IN SENSITIZED WEHI MICE INFECTED WITH LCM OR COWPOX VIRUS.
footpad with $10^7 \text{LD}_{50}$ of WE$_3$ strain LCM virus, a dose which results in hypersensitive footpad swelling (see Section IX). Footpad thickness was measured at four, twenty-four and forty-eight hours and the mean percentage increase, in comparison with the normal foot, is shown in Table 12. It can be seen that pertussis vaccine had no significant effect on the degree of hypersensitive footpad swelling in LCM virus infection. In another experiment, twenty mice were immunized with cowpox virus, by the subcutaneous injection of $10^3 \text{pfu}$ of the Brighton strain, in the form of an infected chorioallantoic membrane suspension. After four weeks, half of these mice were injected intraperitoneally with pertussis vaccine and three days later, both groups were injected in the footpad with $10^7 \text{pfu}$ of cowpox virus. The degree of footpad swelling was calculated at twenty-four and forty-eight hours and the results are shown in Table 12. As in the case of LCM virus, pertussis vaccine had no significant effect on the expression of hypersensitive footpad swelling.

Thus, evidence has been obtained that pertussis vaccine does not alter the cellular immune response to LCM virus, when injected three days after infection.

B. Effects of Freund's Adjuvant on the Response of Mice to LCM Virus Infection

WEHI mice were infected with $10^2 \text{LD}_{50}$ of the WE$_3$
<table>
<thead>
<tr>
<th>ROUTE OF INJECTION</th>
<th>FREUND'S ADJUVANT (0.2 ml. intraperitoneally on day 0)</th>
<th>NUMBER OF MICE TESTED</th>
<th>RESULT</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCM VIRUS (WE&lt;sub&gt;3&lt;/sub&gt; strain, 10&lt;sup&gt;2&lt;/sup&gt;LD&lt;sub&gt;50&lt;/sub&gt; on day 7)</td>
<td>-</td>
<td>18</td>
<td>100% DEATHS AT 7-8 DAYS</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>18</td>
<td>100% DEATHS AT 8-12 DAYS</td>
</tr>
<tr>
<td>INTRACEREBRAL</td>
<td>-</td>
<td>18</td>
<td>20% DEATHS AT 8-12 DAYS</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>18</td>
<td>80% DEATHS AT 8-12 DAYS</td>
</tr>
<tr>
<td>INTRA-PERITONEAL</td>
<td>-</td>
<td>18</td>
<td>FOOTPAD SWELLING AT 7 DAYS. NO SICKNESS.</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>18</td>
<td>FOOTPAD SWELLING AT 7 DAYS. 50% SICKNESS AT 8-12 DAYS.</td>
</tr>
<tr>
<td>FOOTPAD (Subcutaneous)</td>
<td>-</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>12</td>
<td>NO SICKNESS</td>
</tr>
</tbody>
</table>

**Table 13.** EFFECT OF FREUND'S COMPLETE ADJUVANT ON THE RESPONSE OF WEHI MICE TO LCM VIRUS INFECTION.
strain of LCM virus, having received 0.2 ml of Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan) intraperitoneally on the same day, seven days earlier or fourteen days earlier. The only effects seen were when the adjuvant was injected seven days before LCM virus, and the results from these groups of mice are shown in Table 13. In the mice injected with Freund's adjuvant, there was a delay in mortality after intracerebral infection with LCM virus and an increase in mortality after intraperitoneal infection. After footpad infection, some of the adjuvant-treated mice showed obvious signs of sickness, but no deaths occurred and there was no significant difference in footpad swelling.

To investigate whether Freund's adjuvant affected the growth of virus in the viscera, the following experiment was done. Six mice were injected intraperitoneally with Freund's adjuvant and seven days later, along with six control mice, they were infected intraperitoneally with LCM virus. Seven days after infection, titration of pooled livers from the two groups of mice showed that the virus had grown to the same extent in the adjuvant-treated mice \(10^{6.4} \text{LD}_{50} \text{ per gram}\) as in the control mice \(10^{6.6} \text{LD}_{50} \text{ per gram}\). Unlike pertussis vaccine, therefore, Freund's adjuvant did not enhance the
growth of LCM virus in the liver.

Thus, the effects of Freund's complete adjuvant on the response of mice infected by different routes with LCM virus are similar to those of pertussis vaccine, but are less marked.

DISCUSSION

Accumulated evidence suggests that the cellular immune response is the principal factor causing the death of mice infected with LCM virus (Hotchin, 1962a). After intracerebral infection, the mice die in convulsions, indicating that the immune response to growth of virus in the central nervous system is the precipitating factor. In mice infected in the footpad with LCM virus, evidence has been obtained that the cellular immune response plays a part in footpad swelling (see Section IX). Pertussis vaccine and Freund's adjuvant may increase the cellular immune response (White, 1967), but there is evidence that in some circumstances pertussis vaccine causes a decrease in this response (Floersheim, 1965, 1967; Hirano et al., 1967). No significant effect of pertussis vaccine on the hypersensitive footpad response to LCM virus or to cowpox virus could be demonstrated. However, the time of injection of an adjuvant, in relation to the injection
of antigen, is of major importance in determining the
effect of the adjuvant. For example, when Freund's
adjuvant was injected fourteen days before infection or
at the same time as the virus, no effects were noted, but
injection of adjuvant seven days before infection caused
marked effects. It therefore remains a possibility that
in acute infection with LCM virus, pertussis vaccine and
Freund's adjuvant cause a decrease or delay in the
cellular immune response. This would account for the
delayed mortality caused by these agents in mice infected
intracerebrally with LCM virus, and for the delayed and
decreased footpad swelling caused by pertussis vaccine
in mice infected in the footpad.

However, a contradiction is implicit in the
finding that in intraperitoneally infected mice,
treatment with pertussis vaccine or Freund's adjuvant
causes an increase in mortality. In the case of pertussis
vaccine, a possible explanation is as follows. Pertussis
vaccine is known to cause a marked lymphocytosis, which
is maximal two to four days after injection (Morse,
1965), and there is evidence of a great reduction in the
normal recirculation of lymphocytes, in which they pass
from blood to lymph and return to the blood, mainly via
the thoracic duct (Morse and Riester, 1967). In so far
as there is a reduction in the number of mononuclear cells leaving the blood of infected mice after injection of pertussis vaccine, the entry of sensitized lymphoid cells into the central nervous system, or into the tissues of the foot, may be reduced. On the other hand, in tissues in which the parenchymal cells line blood sinusoids, such as the liver and spleen, there may be an increased cellular hypersensitivity reaction, due to the greater numbers of lymphoid cells present in the blood. This may account for the delayed mortality following intracerebral infection and the decreased footpad swelling after footpad infection, and for the increased mortality observed when the predominant infection was in the visceral organs. It has been found that lymphoid cells adhere to vascular endothelium in the infected liver (see Section IV). The retention of sensitized lymphoid cells in infected visceral tissues probably accounts for the reduction in the output of cells in the thoracic duct lymph observed in intravenously-infected mice (Wallnerova, Z., unpublished data).

In mice treated with pertussis vaccine, there was a decreased resistance to the spread of LCM virus and its growth in the viscera was enhanced, as evidenced by the increase in virus titres in the liver following intracerebral,
intraperitoneal or footpad infection. The spread of infection is probably controlled by the antibody response, and it is possible that the antibody response to LCM virus is depressed or delayed by pertussis vaccine, permitting a greater spread of infection via the bloodstream. However, when pertussis vaccine was injected three days after sheep red blood cells, the antibody response was not affected. Experiments need to be done to define the effect of pertussis vaccine on the antibody response to LCM virus. The greater spread of infection helps to explain the increased mortality after intraperitoneal and footpad infection in mice receiving pertussis vaccine. Mims, C.A. (unpublished data) has found that in mice infected intraperitoneally with LCM virus, mortality is increased by splenectomy, which depresses the antibody response.

The antibody response and the cellular immune response probably both play a part in footpad swelling after local LCM virus infection (see Section IX). In mice treated with pertussis vaccine, death after footpad infection was associated with the absence of footpad swelling and sickness with recovery was associated with delayed and diminished footpad swelling. These findings could be explained by a decrease or delay in the antibody response,
as well as in the cellular immune response, to LCM virus. Mims (1969 b) found that treatment with less potent anti-
lymphocyte serum prevented footpad swelling in mice
infected in the footpad with LCM virus, and this was
associated with an increased mortality. Similarly,
when mice infected in the footpad were treated with the
antimetabolite amethopterin, footpad swelling was
prevented and the normally non-lethal infection became
lethal (Sikora & Hotchin, 1962).

Other activities of pertussis vaccine might
play a significant part in the mechanism of its effects
on the response of mice to LCM virus infection.
Pertussis vaccine causes increased sensitivity to stress,
such as exposure to cold, anoxia or X-rays (Kind &
Gadsden, 1953; Rowen et al., 1955; Munoz & Schuchardt,
1957), as well as possibly acting as a stresser agent
itself (Chedid & Boyer, 1956). Also, Bordetella pertussis
organisms contain an endotoxin (Wood, 1940) and LCM virus
infection is known to cause increased sensitivity to
bacterial endotoxins (Barlow & Hotchin, 1963); perhaps
bacterial endotoxins cause increased susceptibility to
LCM virus infection. It may be noted that Suter & Kirsanow
(1961) found an increased sensitivity to endotoxin in
mice infected with mycobacteria, so it is possible that
Freund's complete adjuvant has a similar effect. However,
these activities of pertussis vaccine are unlikely to provide the whole explanation of its effects in mice infected with LCM virus.

SUMMARY

The effects of Bordetella pertussis vaccine and Freund's complete adjuvant on the response of mice to infection with LCM virus depended on the route of infection. In mice infected intraperitoneally with the WE₃ strain of LCM virus, pertussis vaccine caused earlier and increased mortality. After footpad infection, it delayed and decreased footpad swelling and often caused the normally non-lethal infection to become lethal. When mice were infected intracerebrally, pertussis vaccine caused a delay in mortality. There was a decreased host resistance to the spread of virus and its growth in the viscera was enhanced. Freund's complete adjuvant had similar, but less marked, effects on the response of mice to infection by the various routes. Possible mechanisms for the effects of these agents on the response to LCM virus infection have been discussed. However, too little is known about their action on humoral and cellular immune responses for definitive explanations to be made.
IX. THE RESPONSE OF MICE TO THE FOOTPAD INJECTION OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS

In mice, footpad swelling following the local injection of certain poxviruses is a sensitive indicator both of primary infection and of hypersensitivity to these viruses (Fenner, 1948, 1949 a; Schell, 1960 a; Subrahmanyan, 1968). The footpad response of mice to primary infection with LCM virus has been described by Hotchin (1962 b), Hotchin & Benson (1963) and Roger (1963). The mechanisms causing disease and death in animals infected with LCM virus have not been clearly defined, but experimental evidence incriminates the immune response (Rowe, 1954; Hotchin, 1962 a). Since immunological mechanisms may be involved in footpad swelling after local injection of LCM virus, an investigation has been made of the factors contributing to the footpad reaction. The footpad swelling in response to primary footpad infection has been studied and a hypersensitivity response to footpad challenge in previously infected mice has been described and characterized.

RESULTS

A. Primary Footpad Injection of LCM Virus

An inoculum of 0.03 ml, containing $10^2 LD_{50}$ of the WE$_3$ strain of LCM virus, was injected subcutaneously into a hind footpad of six WEHI mice. Footpad measure-
Figure 48. Primary and hypersensitive footpad swelling in WEHI mice following footpad injection of WE$^3$ strain LCM virus.
Figure 49. Footpad at nine days after subcutaneous injection in the footpad with $10^2\text{LD}_{50}$ of WE$_3$ strain LCM virus. Oedema and cellular infiltration. Haematoxylin and eosin. X 40.
ments were taken at daily intervals and the mean percentage increases in thickness of the infected feet are shown in Figure 48. Footpad swelling began at seven days, became maximal at nine days and declined over the next three weeks. The swelling was not confined to the footpad, but involved the whole foot and extended up the leg. The mice did not become sick and no deaths occurred. However, they developed immunity, as shown by resistance to intracerebral challenge with $10^5\text{LD}_{50}$ of LCM virus, ten days after the footpad infection.

Immunofluorescence examination of the feet at nine days after injection showed the presence of viral antigen mainly in the dermis and subcutaneous tissues. Infection was also present in some areas of the epidermis, in some hair follicles and sebaceous glands and in connective tissue around the muscles, but no infected muscle fibres were seen. Many of the infiltrating cells were infected and many contained immunoglobulin.

Histological examination of the feet at nine days showed gross oedema of the dermis and subcutaneous tissues of the footpad and even greater oedema on the dorsum of the foot. There were large numbers of cells infiltrating the dermis and subcutaneous tissues (Figure 49), predominantly in the plantar and lateral areas, with a moderate cellular infiltration of the muscle and
Figure 50. Subcutaneous tissue of footpad at nine days after subcutaneous injection in the footpad with $10^2 LD_{50}$ of WE$_3$ strain LCM virus. Oedema, cellular infiltration and necrotic changes. Haematoxylin and eosin. X 250.
relatively few cells on the dorsum of the foot. The infiltrating cells were mostly large mononuclears and polymorphonuclear leukocytes, with some small lymphocytes and occasional plasma cells. Many of the large mononuclear cells were pyroninophilic. Some of the infiltrating cells were necrotic and others were undergoing mitosis. Deposits of an extracellular homogeneous eosinophilic material were present, mainly in the deeper parts of the subcutaneous tissues, in association with large numbers of infiltrating cells (Figure 50). Scattered karyorrhectic debris was present in these areas. The eosinophilic material was fibrinoid in type, having the same staining characteristics as the deposits seen in the spleen and lymph nodes after systemic LCM virus infection (see Section VI). It was PAS positive, stained reddish-brown with PTAH and did not stain with thioflavine T or Van Gieson's stain.

Factors Involved in the Production of Primary Footpad Swelling

The popliteal lymph node at nine days showed marked oedema and lymphoid cell depletion. There was widespread fibrinoid necrosis and scattered nuclear debris was present. Since these changes in the popliteal lymph node might be expected to affect the lymphatic drainage of the foot, the following experiment was done. An amount of
0.03 ml of India ink was injected into the swollen footpad and into the footpad of normal mice as controls. Three hours later, the popliteal lymph nodes were removed and examined histologically. The control lymph nodes were densely black and contained large amounts of India ink in the afferent lymphatics and medullary sinus, extending into the medullary cords. In the infected mice, India ink had entered the afferent lymphatics, but little or none had entered the node. Thus, there was impedance of the lymphatic drainage of the foot, and this would be one factor contributing to footpad swelling.

An experiment was done to determine the extent of virus growth in the foot and local lymph node after footpad infection with LCM virus. Six WEHI mice were injected in the footpad with $10^2 LD_{50}$ of WE3 strain virus and at six days, the day before swelling would have occurred (see Figure 48), the infected feet and popliteal lymph nodes were removed and titrated. In the foot, the virus titre was $10^{5.2} LD_{50}$ per gram and in the popliteal lymph node it was $10^{4.0} LD_{50}$ per gram. The oedema and fibrinoid necrosis which developed in the footpad and popliteal lymph node probably resulted from the immune response to infection in these tissues (see Section VI). The obstruction to the lymphatic drainage of the foot was
therefore indirectly due to the immune response.

Even with very large doses of LCM virus, primary footpad swelling did not occur before six days after injection. Thus, when the footpad infection consisted of a dose of $10^5 \text{LD}_{50}$ of WE$_3$ strain LCM virus, one-third of the mice developed footpad swelling at six days. A dose of $10^7 \text{LD}_{50}$ caused the death of more than half of the mice, from six to eight days after injection. The footpads of the mice that died showed little or no swelling, but the survivors developed marked footpad swelling at six days, with a maximum at eight days. The time at which primary footpad swelling occurs (Figure 48) suggests that the immune response is involved in the reaction, as after intracerebral or intraperitoneal infection. No footpad swelling occurred when a dose of $10^5 \text{LD}_{50}$ of WE$_3$ strain virus was injected into the footpad of adult LCM virus carrier mice, which are immunologically tolerant to the virus, or into the footpad of newborn mice, which then develop immunological tolerance. An experiment was done to determine the effect of antibody on the development of primary footpad swelling after footpad infection with LCM virus. Twelve mice were injected in the footpad with $10^2 \text{LD}_{50}$ of WE$_3$ strain virus and at five days, half received an intravenous injection
Figure 51. Hypersensitive footpad swelling in WEHI and C$_{57}$BL mice following footpad injection of $10^7$LD$_{50}$ of WE$_3$ strain LCM virus at intervals after initial intravenous injection of $10^2$LD$_{50}$. 
of 0.5 ml guinea pig hyperimmune antiserum to LCM virus. Footpad swelling did not occur earlier in the antiserum-treated mice. In both groups, footpad swelling appeared at seven days and the degree of swelling was identical. Thus, it appears that the presence of (heterologous) antibody in association with infected cells does not by itself cause footpad swelling.

B. Footpad Injection of LCM Virus in Sensitized Mice

To eliminate the possible development of hypersensitivity to host proteins in the virus preparation, different preparations were used for the initial and the test inoculations. WEHI and C57BL mice were injected intravenously with $10^2$LD$_{50}$ of the WE$_3$ strain of LCM virus, in the form of tissue culture fluid from primary mouse embryo fibroblast cell monolayers in which the virus had been passaged once. At two-day intervals until the sixteenth day, groups of six mice of each strain received a dose of $10^7$LD$_{50}$ of a guinea pig lung preparation of WE$_3$ strain virus, injected subcutaneously into a hind footpad. The feet were measured at twenty-four, forty-eight and seventy-two hours and the mean percentage increase in footpad thickness at twenty-four hours is charted in Figure 51. In no case did footpad swelling occur until eight days after the intravenous injection of virus. The
Figure 52. Footpad at twenty-four hours after challenge injection of $10^7$LD$_{50}$ of WE$_3$ strain LCM virus in the footpad, ten days after primary intravenous injection of $10^2$LD$_{50}$. Oedema, cellular infiltration and fibrinoid deposits. Haematoxylin and eosin. X 100.
hypersensitive footpad swelling, which was found to reach a peak at twenty-four hours after the footpad injection, was maximal when this injection was given at ten days after intravenous infection in WEHI mice (see also Figure 48) and at twelve days in C57BL mice. The footpad response rapidly diminished in intensity. By sixteen days after intravenous infection it was only about half the maximal level, and a further reduction had occurred by forty days.

It was found that the footpad hypersensitivity reaction could also be elicited when the mice were initially infected by a route other than the intravenous one - for example, intraperitoneally or subcutaneously in the footpad - or when the Armstrong strain of LCM virus was used for the primary infection (see Section X). In WEHI mice injected in the footpad with $10^7 \text{LD}_{50}$ of LCM virus ten days after intravenous sensitization, the feet were examined histologically at twenty-four hours. The appearances were similar to those in the feet of WEHI mice nine days after a primary footpad infection with LCM virus (see page 129). There was marked oedema of the dermis and subcutaneous tissues of the foot and an extensive cellular infiltration had developed (Figure 52), mostly large mononuclear cells and polymorphonuclear leukocytes.
Figure 53. Hypersensitive footpad swelling following footpad injection of $10^7 \text{LD}_{50}$ of WE3 strain LCM virus in sensitized WEHI mice.
Deposits of fibrinoid material were present amongst collections of the infiltrating cells (Figure 52).

Factors Involved in the Production of Hypersensitive Footpad Swelling

(i) Biphasic Nature of the Response

The time course of the accelerated footpad response suggested that it was predominantly a delayed type hypersensitivity reaction. However, to determine the extent of participation of an immediate, Arthus-type reaction, it was necessary to examine the evolution of the footpad swelling during the first twenty-four hours. Groups of six WEHI mice were injected intraperitoneally, or subcutaneously in the right footpad, with $10^2 LD_{50}$ of WE$_3$ strain LCM virus. Ten days later, a dose of $10^7 LD_{50}$ of the virus was injected into the left footpad of these mice and also into the footpad of mice hyper-immune to LCM virus and of control, uninfected mice. Footpad measurements were taken two-hourly in the early period after injection and then at intervals until seventy-two hours, and the results are shown in Figure 53. During the first ten hours, the footpad swelling in the mice initially injected intraperitoneally was no different from that due to the inoculum in the control mice. However, the mice which had received the initial injection in the footpad responded to injection of the other footpad
with rapid swelling, reaching a peak at six hours and then declining. In the hyperimmune mice, the immediate swelling of the footpad reached a peak at two hours and then declined. Between eighteen and twenty-four hours, all groups of sensitized mice showed a marked increase in footpad swelling. This delayed footpad swelling reached maximal levels at twenty-four hours in mice which had received the initial injection intraperitoneally or in the footpad, but increased gradually to forty-eight hours in the hyperimmune mice. Thus, the footpad hypersensitivity response to LCM virus has a biphasic pattern. The predominant swelling is a delayed type hypersensitivity response, becoming manifest later than eighteen hours after inoculation and reaching a peak between twenty-four and forty-eight hours. In some sensitized mice, probably depending on the level of humoral antibodies, an immediate reaction occurs and becomes maximal in two to six hours.

(ii) Need for Infectious Virus as Inducer

Large amounts of LCM viral antigens appear to be necessary to evoke the footpad hypersensitivity response. In mice which had received an intraperitoneal injection of $10^2LD_{50}$ of WE$_3$ strain virus ten days previously, footpad injection of $10^7LD_{50}$ of the virus caused a fifty
per cent increase in footpad thickness at twenty-four hours (Figure 53), but injection of $10^6\text{LD}_{50}$ into the footpad gave rise to only a fifteen per cent increase at twenty-four hours and $10^5\text{LD}_{50}$ caused no response.

The following experiment was done to determine whether infectious virus was necessary for the production of the footpad hypersensitivity reaction. A suspension containing $10^7\text{LD}_{50}$ of WE$_3$ strain LCM virus per 0.03 ml was heated at $56^\circ\text{C}$ for 30 minutes, thus rendering the virus non-infectious, as tested by intracerebral injection in mice. This material was injected into the footpad in mice hyperimmune to LCM virus and in normal control mice. The feet were measured at two, twenty-four and forty-eight hours, and it was found that although footpad swelling occurred at two hours in the hyperimmune mice, there was no response at twenty-four hours or later. Thus, although the viral antigens present were sufficient to cause the immediate phase of the hypersensitive footpad swelling, the delayed phase apparently required infectious virus for its production. A possible explanation is that LCM virus injected into the footpad of sensitized mice needs to multiply locally, at least to a limited extent, in order to provide sufficient antigen to elicit the delayed hypersensitivity response. It may
be noted in Figure 53 that in the control mice, which received only a footpad injection of $10^7\text{LD}_{50}$ of WE₃ strain virus as a guinea pig lung preparation, the footpad thickness had not returned to its initial level by seventy-two hours. However, in the mice injected in the footpad with a similar preparation in which the virus had been heat-inactivated, the footpad also remained slightly swollen at seventy-two hours.

(iii) **Effect of Route of Sensitizing Injection**

A notable finding was that the greatest degree of hypersensitive footpad swelling occurred when the initial injection was given in the footpad (Figure 53). One possible explanation is that growth of LCM virus in the tissues of the foot produces altered cellular antigens and the immune response to these may contribute to the hypersensitive footpad swelling on viral challenge. Infected cells may be altered because cell membranes bear viral antigens, since LCM virus matures by budding from the cell surface (Dalton et al., 1968). The following experiment was done to investigate this possibility. Six mice were injected in the footpad with $10^2\text{LD}_{50}$ of WE₃ strain LCM virus and at six days, the infected feet were ground in a frozen mortar and a suspension was made, which contained $10^5\text{LD}_{50}$ of virus per ml. The uncentrifuged
suspension, which would have contained cell membranes incorporating any altered antigens, was injected into the footpad of six mice hyperimmune to LCM virus. Insufficient virus was injected to cause a hypersensitivity response. The feet were measured at four, twenty-four and forty-eight hours, but no footpad swelling occurred. This suggests that the immune response to altered cellular antigens does not contribute to the hypersensitive footpad reaction. Another possibility is that there is a cross-over influence from the initially infected hind footpad to the challenged hind footpad, perhaps due to an overlap in the obstruction of lymphatic drainage. An experiment was done to test the validity of this explanation. Six mice were infected in the right hind footpad with $10^5 \text{LD}_{50}$ of WE3 strain LCM virus and six mice were infected with the same dose in the right fore footpad. At ten days, both groups received $10^7 \text{LD}_{50}$ of virus in the left hind footpad, and measurements were taken at four, twenty-four and forty-eight hours. The degree of hypersensitive footpad swelling was not significantly different whether the primary infection was in the opposite hind footpad or in the fore footpad. It remains unclear why initial infection in the footpad is more effective than other routes in producing
Table 14. EFFECT OF INTRAVENOUS INJECTION OF LCM VIRUS ON THE HYPERSENSITIVE FOOTPAD RESPONSE TO THE VIRUS IN SENSITIZED MICE.
sensitization of mice to footpad challenge with LCM virus.

(iv) **Effect of Intravenous Antigen**

The time course and biphasic nature of the hypersensitive footpad response to LCM virus (Figure 53) indicates that there is an immediate, Arthus-type reaction in the early stages, followed later by a delayed type hypersensitivity reaction. An experiment was done to investigate the effect of intravenous administration of antigen on the footpad hypersensitivity response to LCM virus. Twelve mice were infected intraperitoneally with $10^4 LD_{50}$ of Armstrong strain LCM virus and at ten days, half received an intravenous injection of $10^7 LD_{50}$ of WE$_3$ strain virus. Two hours later, both groups were injected in the footpad with $10^7 LD_{50}$ of WE$_3$ strain virus. The feet were measured at four, twenty-four, forty-eight and seventy-two hours and the results are shown in Table 14. It can be seen that intravenous antigen significantly depressed the early (four hour) phase of the footpad swelling and also markedly depressed the twenty-four hour response. By forty-eight hours, however, footpad swelling was not significantly different in the two groups of mice, suggesting that when the intravenously-administered antigen had been removed from the circulation, the delayed phase of
<table>
<thead>
<tr>
<th>GROUP</th>
<th>TREATMENT</th>
<th>MEAN PERCENTAGE INCREASE IN FOOTPAD THICKNESS (Time after injection of virus)</th>
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<tr>
<td></td>
<td></td>
<td>4 hours</td>
</tr>
<tr>
<td>A</td>
<td>SERUM† ALONE</td>
<td>27.8 ± 4.0*</td>
</tr>
<tr>
<td>B</td>
<td>SERUM + LCM VIRUS‡</td>
<td>44.0 ± 4.2</td>
</tr>
<tr>
<td>C</td>
<td>CELLS§ ALONE</td>
<td>32.3 ± 9.0</td>
</tr>
<tr>
<td>D</td>
<td>CELLS + LCM VIRUS‡</td>
<td>45.1 ± 4.7</td>
</tr>
<tr>
<td>E</td>
<td>LCM VIRUS ALONE</td>
<td>17.2 ± 2.4</td>
</tr>
</tbody>
</table>

† 0.05 ml. subcutaneously in footpad
‡ $10^7 LD_{50}$ of WE<sub>3</sub> strain virus subcutaneously in same footpad immediately after serum or cells
§ $10 \times 10^6$ spleen and lymph node cells in 0.05 ml. subcutaneously in footpad.

* Standard deviation

**Table 15.** EFFECT OF TRANSFER OF SERUM OR LYMPHOID CELLS FROM SENSITIZED MICE TO THE FOOTPAD OF NORMAL MICE, ACCOMPANIED BY FOOTPAD INJECTION OF LCM VIRUS.
the footpad response could then occur.

(v) Transfer of Reactivity by Immune Lymphoid Cells

Another experiment was designed to show whether the delayed phase of the footpad hypersensitivity response could be transferred by means of sensitized lymphoid cells. Six WEHI mice were infected intraperitoneally with $10^4 LD_{50}$ of Armstrong strain LCM virus and at ten days, the mice were killed and bled and the serum obtained. The spleen and lymph nodes were removed and a cell suspension was made in Puck's saline, as for adoptive immunization (see page 9), and contained $10 \times 10^6$ viable lymphoid cells per 0.05 ml. Groups of six normal WEHI mice were injected subcutaneously in the footpad with 0.05 ml of serum or lymphoid cell suspension, either alone or followed immediately by injection of $10^7 LD_{50}$ of WE$_3$ strain LCM virus into the same footpad. Another group of mice received the virus injection alone. The feet were measured at four, twenty-four, forty-eight and seventy-two hours and the results are shown in Table 15. It can be seen that in mice which had received a footpad injection of sensitized lymphoid cells plus LCM virus (Group D), footpad swelling at twenty-four hours and later was significantly greater than an additive effect which could be attributed to the swelling caused by cells and virus alone (Groups C & E).
At four hours, however, the footpad swelling in mice which had received serum plus virus (Group B) was no greater than an additive effect of swelling caused by serum or virus alone (Groups A and E). As further controls, serum or spleen and lymph node cells from normal mice were injected into the footpad, followed immediately by an injection of LCM virus. At no time was footpad swelling any greater than an additive effect of the swelling caused by injection of the serum or cells and the virus alone. Thus, it appears that the capability for the delayed phase of the hypersensitive footpad response to LCM virus can be transferred to normal mice by means of sensitized lymphoid cells, but not by serum. It might be expected that the immediate phase of the footpad swelling could be transferred by means of serum, but perhaps the antibody content of the serum inoculum (0.05 ml) was insufficient.

DISCUSSION

The mechanism of the footpad swelling which occurs in mice after primary footpad injection of LCM virus is probably a complex one, and is not simply due to growth of virus in the tissues. Even with a very large dose of virus, footpad swelling did not occur before six days. By this time, an immune response to the infection
would have developed and since virus was present in the footpad, a local immunological reaction probably occurred, with exudation of fluid and cells into the tissues. A local reaction in the footpad, especially when the lymphatic drainage is impaired, would be much more obvious than in subcutaneous tissues elsewhere in the body, because of the dependency of the foot and the importance of the lymphatic drainage in removing accumulated tissue fluid. A local reaction was not obtained when LCM virus was injected subcutaneously into the ear or the tail (Hotchin & Benson, 1963).

Both antibody and the cellular immune response may be involved in the primary footpad swelling. When LCM virus is injected into the footpad, local growth occurs and the first antibody produced would react with viral antigen in excess in the footpad. The activity of antigen-antibody complexes is greatest in antigen excess (Ishizaka et al., 1958, 1959) and their formation in the tissues, particularly if in small blood vessels (Cochrane et al., 1959) would result in an immediate type reaction, with local tissue damage. Antibody might also cause tissue damage by reacting directly with viral antigen which is present at the surface of infected cells, since LCM virus matures by budding from the cell surface (Dalton et al., 1968). Wiktor et al. (1968) have shown that cells of
tissue cultures infected with rabies virus are lysed after exposure to antirabies antibody and complement.

In LCM virus infection, the immune response has been incriminated as a cause of tissue damage (Rowe, 1954; Hotchin, 1962a). Although in primary footpad swelling the marked oedema is suggestive of an antigen-antibody type of response, the lymphatic obstruction was probably an important factor. The other histological changes and the slow evolution of the swelling indicate that a cellular hypersensitivity response plays a large part in the reaction. In the case of ectromelia virus, when a small dose is inoculated into the footpad of mice, footpad swelling does not occur until the seventh day and both humoral and cellular immune responses are probably involved (Fenner, 1948). When LCM virus is injected into the footpad of adult LCM virus carrier mice, which are immunologically tolerant to the virus, or into the footpad of newborn mice, which then develop immunological tolerance, there is no immune response to the virus and no footpad swelling occurs. The primary footpad swelling in mice after footpad infection with LCM virus is inhibited by agents which depress the immune response, such as whole-body X-irradiation (Benson, 1962), amethopterin (Sikora & Hotchin, 1962) or antilymphocyte serum (Mims, C.A., unpublished data). There is evidence
that antilymphocyte serum does not prevent the production of antibody to LCM virus and does not inhibit the growth of the virus in tissues (Hirsch et al., 1967, 1968).

Also, it has been shown that the presence of (heterologous) antibody in association with infected cells does not by itself cause footpad swelling. These findings suggest that the cellular immune response makes a more important contribution to the primary footpad swelling than does the antibody response.

The injection of mice with Bordetella pertussis vaccine had the same effect as immunosuppressive treatments and depressed the primary footpad response to LCM virus infection (see Section VIII). It also caused a normally non-lethal infection to become lethal, as occurred after treatment with amethopterin (Sikora & Hotchin, 1962) or less potent antilymphocyte sera (Mims, C.A., unpublished data). It therefore seems that depression of footpad swelling enables LCM virus to spread much more readily from the footpad and cause a greater systemic infection.

Another factor which may play an important part in primary footpad swelling, in response to footpad infection with LCM virus, is impedance of the lymphatic drainage of the foot. In his original description of the primary footpad reaction of mice to LCM virus, Hotchin
(1962 b) referred to a "severe obliterative lesion" in the local lymph node. When mice were injected in the footpad with WE₃ strain LCM virus, the popliteal lymph node became infected, fibrinoid necrosis developed and the node was reduced to a necrotic, eosinophilic reticulum (see also Section VI). When India ink was injected into the swollen foot, the ink filled the afferent lymphatics, but there was almost total inhibition of its entry into the swollen necrotic node. Similar observations have been made by Mims (1968 a) in the case of cowpox virus infection of the footpad in mice. The obstruction of lymphatic drainage to the popliteal lymph node would contribute to the swelling of the footpad. Perhaps there is similar lymphatic obstruction at the level of the inguinal nodes, to account for the oedema of the leg. When the footpad is infected with the Armstrong strain of LCM virus, which does not cause necrosis of lymphoid tissues (see Section VI), oedema also occurs, though to a lesser extent than with WE₃ strain virus (see Section X). When India ink is injected into the footpad swollen after infection with Armstrong strain virus, there is impedance of its entry into the popliteal lymph node (see Section X), indicating that even in the absence of necrotic changes in lymphoid tissues, some degree of lymphatic obstruction can occur.
It has been demonstrated that the latent period before primary footpad swelling after footpad infection with LCM virus (Roger & Roger, 1964b), or death after intracerebral infection (Rowe, 1954; Seamer et al., 1963), can be shortened by a previous injection of the virus several days earlier. However, the footpad hypersensitivity response to LCM virus has not previously been described. The early peak in hypersensitive footpad swelling (Figure 53) is consistent with an Arthus-type response, which normally reaches maximal levels three to six hours after injection (Crowle, 1959a; Leskowitz & Waksman, 1960). The time course of the second phase of the footpad hypersensitivity response to LCM virus, with swelling commencing later than ten hours and reaching maximal levels at twenty-four to forty-eight hours, suggests that it is a cellular hypersensitivity response (O'Grady, 1957; Crowle, 1959a; Uhr, 1966). In mice immune to ectromelia virus, hypersensitive footpad swelling reaches maximal levels in twelve to twenty-four hours (Fenner, 1948, 1949a, b) and appears to be due to a cellular hypersensitivity response. However, the presence of early swelling, and the participation of antibody in this, has not been excluded. The hypersensitive footpad response to LCM virus was greatest when the primary infection
was in the footpad (Figure 53), but the explanation for this is not apparent. In many experimental systems, it has been shown that the most effective routes of injection for the induction of delayed hypersensitivity are the intradermal or subcutaneous (in particular the footpad) routes (Leskowitz & Waksman, 1960; Uhr, 1966).

In mice which had been sensitized to LCM virus, it was found that intravenous injection of virus caused a depression of the hypersensitive footpad response to subsequent footpad injection of the virus. The footpad swelling was reduced both at four hours and at twenty-four hours. It is well known that immediate hypersensitivity, which is mediated by antibodies, may be temporarily depressed by intravenous injection of the specific antigen (Weiser et al., 1941; Morgan et al., 1957). Delayed hypersensitivity may also be depressed by the systemic injection of antigen (Uhr & Pappenheimer, 1958; Oliveira-Lima, 1958). It is a characteristic of delayed type hypersensitivity that it can be passively transferred to normal animals by injection of living sensitized lymphoid cells, but not by serum (Crowle, 1959a; Uhr, 1966; Benacerraf & Green, 1969). When spleen and lymph node cells from mice sensitized to LCM virus were injected into the footpad of normal mice,
followed immediately by injection of the virus, footpad swelling occurred at twenty-four hours, but was not present at four hours. The injection of serum along with the virus did not cause this reaction, nor did the lymphoid cells alone. This finding provides further evidence that the second phase of the hypersensitive footpad response to LCM virus is a delayed type hypersensitivity response.

Thus, it has been shown that footpad swelling after local injection of LCM virus is not only a sensitive indication of primary infection, as also shown by Hotchin (1962 b), Hotchin & Benson (1963) and Roger & Roger (1964 a), but in addition provides evidence of a state of hypersensitivity to the virus, predominantly a cellular type of hypersensitivity. In both the primary and the hypersensitive footpad reactions, there are similar histological changes, with oedema and cellular infiltration and the formation of deposits of fibrinoid material, the pathogenesis of which is discussed in Section VI. It is probable that the footpad reactions to LCM virus are local manifestations of the mechanisms causing disease in systemic LCM virus infection.

SUMMARY

The local responses to injection of LCM virus into
the footpad of mice have been studied. Primary footpad infection results in swelling of the foot and the factors contributing to this reaction have been investigated. The immune response to the virus, as well as obstruction to the lymphatic drainage of the foot, appear to be involved. In mice previously infected with LCM virus, a hypersensitivity reaction has been demonstrated in the footpad on local challenge with the virus. The reaction is biphasic, with an immediate response followed by delayed footpad swelling, pointing to the participation of both humoral and cellular immune responses, with the latter predominating. The delayed phase of the hypersensitive footpad swelling could be passively transferred to normal mice by injection of lymphoid cells from sensitized donors into the footpad, immediately prior to challenge with virus. It is suggested that the mechanisms of the footpad reactions are identical with those causing lesions in other tissues infected with LCM virus.
X. THE RESPONSE OF MICE TO INFECTION WITH DIFFERENT STRAINS OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS

No antigenic differences have been demonstrated between strains of LCM virus, but the passage history has been shown to be of great importance in determining the behaviour of the virus in animals (Traub, 1937; Schwartzman, 1946). A number of workers have compared virus growth and mortality in adult and in infant mice infected by various routes with different strains of LCM virus (Rowe, 1954; Traub, 1960 b; Hotchin & Weigand, 1961 a; Hotchin & Benson, 1963; Lehmann-Grube, 1964 a, b). In the present studies, using the WE₃ and Armstrong strains of LCM virus, this work has been extended. Immunofluorescence and histological examination have been used as well as titration methods, and possible reasons for the difference in behaviour of the two strains of virus have been investigated.

RESULTS

It was found that a small dose ($10^2\text{LD}_{50}$) of the WE₃ strain of LCM virus was lethal for guinea pigs when injected intraperitoneally or subcutaneously in the flank or in the footpad. However, even larger doses ($10^5\text{LD}_{50}$) of the Armstrong (mouse-adapted) strain virus injected by
<table>
<thead>
<tr>
<th>ROUTE OF INFECTION</th>
<th>WE₃ STRAIN VIRUS</th>
<th>ARMSTRONG STRAIN VIRUS</th>
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<tr>
<td>INTRACEREBRAL</td>
<td>100% deaths</td>
<td>100% deaths</td>
</tr>
<tr>
<td></td>
<td>Median Survival Time = 8 days</td>
<td>Median Survival Time = 7 days</td>
</tr>
<tr>
<td>FOOTPAD (Subcutaneous)</td>
<td>No deaths</td>
<td>No deaths</td>
</tr>
<tr>
<td></td>
<td>Footpad swelling at 7 - 30 days</td>
<td>Footpad swelling (less) at 9 - 24 days</td>
</tr>
<tr>
<td>INTRAPERITONEAL</td>
<td>20% deaths</td>
<td>No deaths</td>
</tr>
<tr>
<td></td>
<td>Median survival time = 10 days</td>
<td>Growth +</td>
</tr>
<tr>
<td></td>
<td>Growth ++</td>
<td>No pleural or peritoneal fluid</td>
</tr>
<tr>
<td></td>
<td>Pleural and Peritoneal fluid + +</td>
<td></td>
</tr>
<tr>
<td>INTRAVENOUS</td>
<td>No deaths</td>
<td>No deaths</td>
</tr>
<tr>
<td></td>
<td>Growth ++</td>
<td>Growth +</td>
</tr>
</tbody>
</table>

**Table 16.** Response of Adult WEHI Mice to Infection by Various Routes with $10^2LD_{50}$ of Different Strains of LCM Virus
these routes caused no sickness or death in guinea pigs, but resulted in immunity, as determined by challenge with $10^6 \text{LD}_{50}$ of WE$_3$ strain virus intraperitoneally ten days later.

The following experiments demonstrate the different effects of the two strains of LCM virus in mice. Adult WEHI mice were injected by various routes with the WE$_3$ or the Armstrong strain of virus and median survival times were calculated from the mortality observed in more than fifty mice. For immunofluorescence, histological examination and estimation of virus titres, groups of six mice were used.

**Intracerebral Injection of LCM Virus**

A dose of $10^2 \text{LD}_{50}$ of WE$_3$ or Armstrong strain virus was injected intracerebrally in mice and the results are shown in Table 16. With either strain of virus, all the mice died in convulsions, with hind limbs extended, but the median survival time was less with Armstrong strain virus infection. With WE$_3$ strain virus, no deaths occurred until seven days, but with Armstrong strain virus, some mice died at six days.

At six days after injection, frozen sections of the tissues were stained with fluorescein-conjugated antiserum against LCM virus. No significant differences
were detected in the pattern or extent of central nervous system (CNS) infection with either strain of virus. Infection was limited to the meninges, choroid plexuses, ependyma and Virchow-Robin spaces, with no extension into the brain substance, apart from some infected cells in submeningeal and subependymal areas. By routine histological examination, mice infected with either strain of virus showed a marked mononuclear cell infiltration in the meninges, choroid plexuses and some Virchow-Robin spaces. The infiltrating cells were mostly large mononuclears, with a small number of small lymphocytes and few if any plasma cells and polymorphonuclear leukocytes. The liver and spleen showed marked infection with the $WE_3$ strain virus, but in mice injected with the Armstrong strain virus, no infected cells were seen. By routine histology, no pathological changes were seen in the liver and spleen after injection of Armstrong strain virus, but with $WE_3$ strain virus the following changes were present. The liver showed hepatic cell vacuolation and a moderate mononuclear cell infiltration in the portal tracts and sinusoids. Scattered necrotic hepatic cells were present, singly and in numerous small foci, surrounded by mononuclear cells. The infiltrating cells were mainly large mononuclears, with small numbers of small lymphocytes, plasma cells and polymorphonuclear leukocytes. In the spleen, there were many large pale mononuclear cells surrounding the
<table>
<thead>
<tr>
<th>STRAIN OF VIRUS</th>
<th>VIRUS TITRE ($\log_{10}^{\text{LD}_{50}}$ per gram)</th>
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<tr>
<td></td>
<td>FIVE DAYS AFTER INTRACEREBRAL INFECTION</td>
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<tr>
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<td>BRAIN</td>
</tr>
<tr>
<td>WE$_3$</td>
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</tr>
<tr>
<td>ARMSTRONG</td>
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**Table 17.** VIRUS TITRES IN TISSUES OF ADULT WEHI MICE AFTER INTRACEREBRAL OR FOOTPAD INFECTION WITH $10^2 \text{LD}_{50}$ of DIFFERENT STRAINS OF LCM VIRUS.
follicles and areas of fibrinoid necrosis (see Section VI) were present in some perifollicular regions, occasionally extending into the centre of follicles.

At five days after infection, suspensions of the brain and spleen were titrated and the results are shown in Table 17. It can be seen that the WE$_3$ strain virus grew in both organs to a much higher titre than the Armstrong strain virus. Thus, the Armstrong strain of LCM virus, although it grew to lower levels in the brain after intracerebral injection, caused mice to die earlier than after intracerebral injection of WE$_3$ strain virus. Since the death of mice infected with LCM virus appears to be due to the immune response (Rowe, 1954; Hotchin, 1962 a), it would seem that the Armstrong (mouse-adapted) strain virus gives rise to an immune response of greater intensity than does the WE$_3$ strain virus. After Armstrong strain virus infection, there is very little growth of virus in the spleen and liver, as detected by assay and by immunofluorescence, and this explains the absence of necrotic lesions in these organs.

**Footpad Injection of LCM Virus**

A dose of $10^2LD_{50}$ of WE$_3$ or Armstrong strain virus was injected subcutaneously into a hind footpad in groups of twelve mice. By this route of injection, no sickness
Figure 54. Primary footpad swelling in WEHI mice after injection in the footpad with $10^2LD_{50}$ of WE$_3$ or Armstrong strain LCM virus.
or deaths occurred. Footpad measurements were taken daily and the results are shown in Table 16 and Figure 54. It can be seen that footpad swelling began later and was less marked with the Armstrong strain virus than with the WE₃ strain virus. At six days after injection, suspensions made from the infected foot and popliteal lymph node were titrated and the virus titres are shown in Table 17. As in the brain and spleen after intracerebral injection, the WE₃ strain virus grew to much higher titres than the Armstrong strain virus.

Immunofluorescence examination of the feet at nine days after injection with either strain of virus showed no difference in the distribution of infection, but with Armstrong strain virus, fewer infected cells were present. There was infection in the dermis and subcutaneous tissues and in some areas of the epidermis. Some infected hair follicles and sebaceous glands were present and infection was also seen in connective tissue around the muscles, but not in the muscle fibres themselves. In the popliteal lymph node and spleen of mice injected with WE₃ strain virus, a moderate number of infected large mononuclear cells were seen, but with Armstrong strain virus, there were less infected cells in the popliteal lymph node and none were detected in the spleen.
At nine days, histological examination of the infected feet showed marked oedema of the dermis and subcutaneous tissues of the footpad and the dorsum of the foot, and this was noticeably greater with the WE₃ strain virus infection. There were large numbers of cells infiltrating the dermis and subcutaneous tissues, predominantly in the plantar and lateral areas, with a moderate cellular infiltration of the muscle and relatively few cells on the dorsum of the foot. The infiltrating cells were mostly large mononuclear cells and polymorphonuclear leukocytes, with some small lymphocytes and occasional plasma cells. In feet infected with WE₃ strain virus, deposits of fibrinoid material were present, mainly in the deeper parts of the subcutaneous tissues (see Section IX), and nuclear debris was scattered in these areas. With Armstrong strain virus, no fibrinoid necrosis was seen and there was little or no evidence of pyknotic or karyorrhectic degeneration of the infiltrating cells. In WE₃ strain virus infection, the popliteal lymph node was oedematous and showed lymphoid cell depletion, with marked fibrinoid necrotic changes and scattered karyorrhectic debris (see also Section VI), and fibrinoid necrosis was also present in the spleen. In Armstrong strain virus infection, the
popliteal lymph node showed oedema and increased cellularity, but no necrotic changes had occurred.

An experiment was done to determine whether obstruction to the lymphatic drainage of the foot occurred after footpad infection with LCM virus. Mice were injected in the footpad with $10^2LD_{50}$ of WE₃ or Armstrong strain virus and at nine days, 0.03 ml of India ink was injected into the swollen footpad and also into the footpad of normal control mice. Three hours later, the popliteal lymph node was examined macroscopically and histologically. In the control mice, the node was densely black and there were large amounts of India ink in the afferent lymphatics and medullary sinus, extending into the medullary cords. In the mice infected with WE₃ strain virus, India ink was present in the afferent lymphatics, but little or none had entered the node. With Armstrong strain virus infection, small to moderate amounts of India ink had entered the medullary sinuses of the popliteal node, but much less than in the control mice. Thus, there is impedance of the lymphatic drainage of the foot after footpad infection with either WE₃ or Armstrong strain LCM virus, and this is probably one factor involved in primary footpad swelling in response to LCM virus infection (see also Section IX).
Figure 55. Hypersensitive footpad swelling in WEHI mice following footpad injection of $10^7 \text{LD}_{50}$ of WE$_3$ strain LCM virus at ten days after intraperitoneal injection of $10^2 \text{LD}_{50}$ of WE$_3$ or Armstrong strain virus.
Since some degree of lymphatic obstruction occurred in Armstrong strain virus infection, necrotic changes in the lymphoid tissues are therefore not essential for its development, and oedema and hypercellularity of the lymph nodes may be sufficient to impede the lymph flow.

When sensitized mice are challenged in the footpad with LCM virus, the hypersensitive footpad swelling is biphasic, depending in turn on an antibody-type response and a delayed type hypersensitivity response (see Section IX). An experiment was done to compare the hypersensitive footpad reaction to challenge following initial infection with either the WE$_3$ or the Armstrong strain of virus.

Groups of ten mice were injected intraperitoneally with $10^2$LD$_{50}$ of WE$_3$ or Armstrong strain virus, grown in mouse embryo fibroblast tissue culture, and at ten days they were injected subcutaneously in the footpad with $10^7$LD$_{50}$ of a guinea pig lung suspension of WE$_3$ strain virus. The feet were measured at four, twenty-four and forty-eight hours and then at intervals until the swelling had disappeared, and the results are shown in Figure 55. It can be seen that both the early (four hour) phase and the late phase of the hypersensitive footpad reaction were more marked after initial infection with the Armstrong strain virus. Thus, both the humoral and the cellular immune
responses appear to be stimulated to a greater extent by the Armstrong strain of LCM virus than by the WE₃ strain virus.

**Intraperitoneal Injection of LCM Virus**

Mice were injected intraperitoneally with $10^2\text{LD}_{50}$ of WE₃ or Armstrong strain virus and the results are summarized in Table 16. With WE₃ strain virus, approximately twenty per cent of the mice died and there were marked pleural and peritoneal effusions, but no convulsions occurred. With Armstrong strain virus, the mice remained healthy and no pleural or peritoneal effusions developed. However, the mice developed immunity to LCM virus, as determined by their resistance to the intracerebral injection of $10^2\text{LD}_{50}$ of WE₃ strain virus, fourteen days later.

At five days, immunofluorescence examination of the spleen and liver showed marked infection with the WE₃ strain virus, but no infected cells were seen in mice injected with the Armstrong strain virus. At six days, routine histological examination of the liver and spleen showed no pathological changes with the Armstrong strain virus, but with the WE₃ strain virus there were similar changes to those described after intracerebral injection of this virus strain (see page 153).

When a larger dose ($10^5\text{LD}_{50}$) of WE₃ strain virus was injected intraperitoneally, all the mice died.
<table>
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<tr>
<th>ROUTE OF INJECTION</th>
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<tr>
<td></td>
<td>BRAIN</td>
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<td>INTRAPERITONEAL</td>
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<tr>
<td>INTRAVENOUS</td>
<td>1.8</td>
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</tbody>
</table>

ND = Not done

Table 18. VIRUS TITRES IN TISSUES OF ADULT WEHI MICE AT FIVE DAYS AFTER INTRAPERITONEAL OR INTRAVENOUS INJECTION OF $10^5LD_{50}$ OF THE ARMSTRONG STRAIN OF LCM VIRUS.
However, even with a dose of $10^5 \text{LD}_{50}$ of Armstrong strain virus, no sickness or effusions developed and at five days, no infected cells were detected in the liver and spleen by immunofluorescence and no pathological changes were seen on histological examination. At five days after intraperitoneal injection, tissue specimens were titrated and the results are shown in Table 18. It can be seen that little or no growth of Armstrong strain virus had occurred in the tissues.

Thus, the Armstrong strain virus did not grow to a significant extent when injected intraperitoneally, but the WE$_3$ strain virus grew in the tissues and some or all of the mice died, depending on the dose of virus injected.

**Intravenous Injection of LCM Virus**

Mice were injected intravenously with $10^2 \text{LD}_{50}$ of WE$_3$ or Armstrong strain virus and the results are shown in Table 16. No sickness or death occurred with either strain of virus. At six days, immunofluorescence examination showed that infection of the liver and spleen had occurred with WE$_3$ strain virus, but not with Armstrong strain virus. There were no infected cells in the brain or meninges, with either strain of virus, and histological examination showed no abnormality in the brain or meninges.
The liver and spleen of mice injected with the WE₃ strain virus showed pathological changes similar to those described after intracerebral injection of this virus strain (see page 153), but there were no changes in mice injected with the Armstrong strain virus.

When a larger dose \(10^5\text{LD}_{50}\) of WE₃ strain virus was injected intravenously, all the mice died. At six days, there was marked infection in the brain and meninges, accompanied by a mononuclear cell infiltration (see also Section V). In the liver and spleen, infection was more extensive and the pathological changes were much more severe than after injection of a small dose of virus (see also Section IV). When a dose of \(10^5\text{LD}_{50}\) of Armstrong strain virus was injected intravenously, no sickness or death occurred. Immunofluorescence and histological examination at six days and eleven days showed no infection or pathological changes in the brain and meninges or in the liver and spleen. At five days after intravenous injection, tissue suspensions were titrated and the results are shown in Table 1.8. Again it can be seen that the Armstrong strain virus showed little or no growth in the tissues.

Thus, the Armstrong strain of LCM virus did not grow to a significant extent in the tissues after
intravenous injection of small or large doses of virus. With the WE$_3$ strain virus, a small dose resulted in growth and pathological changes in the viscera, but was insufficient to cause death. After injection of a large dose, greater virus growth occurred in the viscera and there were more severe pathological lesions, leading to death of the mice. Infection of the CNS was seen after the intravenous injection of a large dose of WE$_3$ strain virus.

**Injection of Carrier Virus**

Carrier virus, obtained from WEHI mice of an LCM virus carrier colony, was found to be similar in its behaviour, in normal adult WEHI mice, to that of the Armstrong (mouse-adapted) strain of LCM virus. When $10^2LD_{50}$ of carrier virus was injected intracerebrally, all the mice died, but after intraperitoneal or intravenous injection the mice remained healthy. At six days, no infected cells were detected in the liver and spleen by immunofluorescence, and histological examination showed no pathological changes in these organs.

Thus, despite the fact that WE$_3$ strain LCM virus was used to initiate the carrier colony, virus which had been transmitted in utero for several generations had undergone a change in character and now behaved like the mouse-adapted Armstrong strain of virus.
<table>
<thead>
<tr>
<th>STRAIN OF VIRUS</th>
<th>BRAIN VIRUS TITRE ($\log_{10}^{LD_{50}}$ per gram)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>INTRAPERITONEAL INJECTION</td>
</tr>
<tr>
<td>WE$_3$</td>
<td>8.6</td>
</tr>
<tr>
<td>ARMSTRONG</td>
<td>6.3</td>
</tr>
</tbody>
</table>

**Table 19.** BRAIN VIRUS TITRES IN INFANT WEHI MICE AT FIVE DAYS AFTER INTRAPERITONEAL OR INTRACEREBRAL INJECTION OF $10^5LD_{50}$ OF DIFFERENT STRAINS OF LCM VIRUS.
Response of Neonatal Mice to Infection with Different Strains of LCM Virus

A dose of $10^5LD_{50}$ of the WE$_3$ strain or the Armstrong strain of LCM virus was injected intraperitoneally in WEHI mice less than twenty-four hours after birth. With the WE$_3$ strain virus, one out of twenty-two mice died, at fifteen days, and with the Armstrong strain virus, twelve out of sixteen mice died, between twelve and eighteen days.

At seven days after injection, immunofluorescence examination showed infection of the liver and spleen with both strains of virus, though more extensive with the WE$_3$ strain virus. With WE$_3$ strain virus, there was extensive infection of the meninges and brain substance, but with Armstrong strain virus, infection was at this time confined to the meninges, choroid plexuses, ependyma and some subependymal and submeningeal cells (see also Section V). By three weeks, however, the Armstrong strain virus had spread throughout the brain substance, to the same extent as the WE$_3$ strain virus. At five days after intraperitoneal injection, the brains were titrated and the results are shown in Table 19. It can be seen that the WE$_3$ strain virus had grown to a much higher titre than the Armstrong strain virus.
To compare the extent of infection of the brain after direct intracerebral inoculation, newborn mice were injected intracerebrally with $10^5\text{LD}_{50}$ of either strain of virus (see also Section V). At five days, the brains were titrated and the results are shown in Table 19. In contrast to the result of intraperitoneal injection, the Armstrong strain virus had grown to the same titre as the WE$_3$ strain virus. At seven days, immunofluorescence staining showed that with both strains of virus, infection was present throughout the meninges and brain substance. The liver and spleen were also infected with both virus strains, but infection was again more marked with the WE$_3$ strain virus. After direct intracerebral injection, therefore, both strains of LCM virus spread rapidly throughout the CNS, whereas after extraneural injection, the WE$_3$ strain virus infects the brain substance much more rapidly than the Armstrong strain virus.

Thus, in neonatal mice, in contrast to infection in adult mice, marked infection of the viscera occurred with the Armstrong strain of LCM virus as well as with the WE$_3$ strain virus. Also, even after extraneural inoculation, both strains of virus caused CNS infection. The mortality was much higher in mice infected with Armstrong strain virus, suggesting that the immune response
Clearance of Different Strains of LCM Virus from the Blood in Adult and Infant Mice

It has been shown that in adult mice, the WE₃ strain of LCM virus causes marked infection of the viscera, whereas the Armstrong strain virus does not cause significant visceral infection (see also Section IV). To test the possibility that Armstrong strain virus is not taken up in significant amounts from the blood of adult mice, the blood clearance of the two strains of virus was compared. Clearance of carrier virus, which has been shown to be similar in its behaviour to Armstrong strain virus (see page 162), was also tested. In addition, clearance was compared in adult and infant mice, because both Armstrong and WE₃ strains of virus cause visceral infection in infant mice.

These experiments are reported in detail in Section IV (see page 39). A known amount of virus was injected intravenously in adult or three-day-old mice and at five, thirty and sixty minutes, serum samples were taken and titrated. A reduction in the level of circulating virus indicated that clearance of the virus had occurred. In summary, about ninety per cent of
<table>
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<th>STRAIN OF VIRUS</th>
<th>VIRUS TITRE IN BLOOD ($\log_{10}^{\text{LD}_{50}}$ per ml.)</th>
<th>VIRUS TITRE IN LIVER ($\log_{10}^{\text{LD}_{50}}$ per gram)</th>
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</thead>
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<td>WE$_3$</td>
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<td>6.2</td>
</tr>
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<td>WE$_3$</td>
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<tr>
<td>INFANT</td>
<td>ARMSTRONG</td>
<td>3.4</td>
<td>3.6</td>
</tr>
</tbody>
</table>

**Table 20.** CLEARANCE AND GROWTH OF WE$_3$ AND ARMSTRONG STRAINS OF LCM VIRUS AFTER INTRAVENOUS INJECTION IN ADULT AND INFANT WEHI MICE.
The WE₃ strain virus was removed from the blood of adult mice within five minutes after intravenous injection, whereas Armstrong strain virus and carrier virus were not cleared to a significant extent in sixty minutes. In infant mice, neither Armstrong nor WE₃ strain virus was detectably cleared from the blood within sixty minutes after intravenous injection. By seven days, however, the liver of infant mice is heavily infected after injection of either strain of virus. Clearance of virus from the blood may have occurred later than sixty minutes, so the clearance experiments were extended in time, comparing adult and infant mice injected intravenously with each strain of virus. Mice were injected intravenously with a known dose of WE₃ strain virus as a guinea pig lung preparation, and Armstrong strain virus as a mouse brain preparation, and the theoretical immediate titre in the blood was calculated. The blood was titrated at sixty minutes and three days, and the liver at three days, and the results are shown in Table 20. In adult mice, the blood virus titres decreased over the three day period, with both strains of virus. The WE₃ strain virus, after clearance from the blood, was present in high titre in the liver by three days, but the Armstrong strain virus, which was not detectably cleared from the blood in...
sixty minutes, had not grown to a significant titre in the liver by three days. In infant mice, in which neither strain of LCM virus was detectably cleared from the blood by sixty minutes, virus titres at three days indicated that both WE₃ and Armstrong strain virus had grown in the liver, with perhaps some seeding of virus into the blood.

In summary, the WE₃ strain virus was rapidly cleared from the blood of adult mice and caused visceral infection, whereas the Armstrong strain virus was not detectably cleared and visceral infection did not occur to a significant extent. In infant mice, visceral infection occurred with both strains of virus, despite the lack of detectable clearance from the blood by sixty minutes.

Infection of Peritoneal Macrophages by Different Strains of LCM Virus

It has been shown that the WE₃ and Armstrong strains of LCM virus both grow in mouse cells in vitro (Wainwright & Mims, 1967). The observation that WE₃ strain LCM virus is rapidly cleared from the blood of adult mice, whereas Armstrong strain virus is not cleared significantly, points to a difference in uptake of the two strains of virus by phagocytic cells.
mouse macrophages in vitro was therefore compared, with the \( \text{WE}_3 \) and Armstrong strains of virus. Ring cultures of mouse peritoneal macrophages were infected with \( 10^4 \text{LD}_{50} \) of either strain of virus, in Eagle's medium containing 20% calf serum. After incubation for forty-eight hours, the cells were dried, fixed and stained with fluorescein-conjugated antiserum against LCM virus. Under these conditions, it was found that forty per cent of the cells were infected with \( \text{WE}_3 \) strain virus and only three per cent with Armstrong strain virus. To determine the effect of mouse serum, instead of calf serum, on the in vitro infection of mouse peritoneal macrophages, ring cultures were collected and maintained in Eagle's medium containing 20% normal mouse serum. After infection of the cells with \( 10^4 \text{LD}_{50} \) of \( \text{WE}_3 \) or Armstrong strain virus, immunofluorescence examination at forty-eight hours showed that forty per cent of the cells were infected with \( \text{WE}_3 \) strain virus and one per cent with Armstrong strain virus. Thus, peritoneal macrophages in vitro are much less readily infected by the Armstrong strain of LCM virus than by the \( \text{WE}_3 \) strain virus.

To examine the direct infection of peritoneal cells in the intact animal, the following experiment was performed.
A dose of $10^4\text{LD}_{50}$ of WE$_3$ or Armstrong strain virus was injected intraperitoneally in adult mice. At forty-eight hours, peritoneal washings were done and the cells collected were transferred to rings on glass slides and incubated for two hours, to enable macrophages to adhere to the glass. Non-adherent cells were then washed off and the macrophages were stained with fluorescein-conjugated antibody to LCM virus. Infection was detected in seventy per cent of the cells obtained from mice injected with WE$_3$ strain virus, but in only four per cent of the cells from mice injected with Armstrong strain virus.

Thus, mouse peritoneal macrophages appear to be much less readily infected by the Armstrong strain of LCM virus than by the WE$_3$ strain virus, in vivo as well as in vitro.

DISCUSSION

Death in mice infected with LCM virus is probably due to the immune response to the virus (Rowe, 1954), in particular the cellular immune response (Hotchin, 1962 a). In mice infected with the Armstrong strain of LCM virus, there appears to be a greater immune response than with WE$_3$ strain virus infection. It was found that in adult mice, the median
survival time was less after intracerebral infection with Armstrong strain virus (Table 16), even though \( \text{WE}_3 \) strain virus grew to a higher titre in the brain and meninges (Table 17). Also, the hypersensitive footpad reaction to LCM virus is caused by the immune response (see Section IX), and it was found that hypersensitive footpad swelling was greater after footpad challenge in mice which had initially been infected with Armstrong strain virus (Figure 55). This is despite the fact that \( \text{WE}_3 \) strain virus grows to higher titres in the tissues and so would be expected to provide a greater antigenic stimulus during the initial infection. In addition, there is a greater mortality in Armstrong strain virus infection of neonatal mice (see page 163; Whitney, 1951; Lehmann-Grube, 1964 b), and this is consistent with the appearance of a more vigorous immune response in these infant mice, because it is the immune response which may lead to death rather than immunological tolerance (Hotchin & Cinitis, 1958; Hotchin & Weigand, 1961 a).

After primary footpad infection, \( \text{WE}_3 \) strain virus grew in the foot to a higher titre than Armstrong strain virus (Table 17), and footpad swelling was earlier in onset and greater in degree with the \( \text{WE}_3 \) strain
virus infection (Figure 54). It has been shown that the immune response plays an important part in primary footpad swelling (see Section IX) and a greater immune response might be expected in Armstrong strain virus infection. However, obstruction of the lymphatic drainage of the foot was found to be more marked with WE3 strain virus infection and this would result in greater footpad swelling. Also, since the WE3 strain virus grows to a higher titre in the foot, perhaps there is a more marked local Arthus-type reaction, leading to greater oedema and possibly an earlier onset of footpad swelling.

Shwartzman (1946) showed that strains of LCM virus maintained by mouse passage or by guinea pig passage were both lethal for mice after intracerebral injection, but after extraneural injection only the guinea pig passaged virus caused death of the mice. When mice were injected intracerebrally with the WE3 or the Armstrong strain of LCM virus, both grew in the CNS (see also Lehmann-Grube, 1964 a) and the probable cause of death was a local cellular immune reaction. After intravenous or intraperitoneal injection, however, Armstrong strain virus did not grow to a significant extent in the tissues, so tissue damage due to a
cellular hypersensitivity reaction would not be expected to occur. With WE₃ strain virus, there was marked infection in the viscera and necrotic lesions occurred in the liver and spleen, probably due to the cellular hypersensitivity response (see Section VI). It has been shown that after growth of WE₃ strain virus has occurred in the viscera, the virus spreads via the bloodstream to the CNS (see Section V). When mice were infected extraneurally with Armstrong strain virus, there were only low levels of virus in the viscera and the blood (Table 18; Lehmann-Grube, 1964 a), and this would account for the failure of the virus to infect the CNS. In infant mice injected extraneurally, both strains of virus grew in the viscera (Table 20) and infection of the CNS occurred with the Armstrong strain virus as well as with the WE₃ strain virus (Table 19). Infection spread throughout the brain substance in infant mice, but not in adult mice, and possible explanations for this have been discussed in Section V.

The experiments with mouse peritoneal macrophages indicate that WE₃ strain virus infects these cells much more readily than Armstrong strain virus, both in vitro and in vivo. Also, it has been found that intravenously injected WE₃ strain LCM virus is rapidly cleared from the
blood of adult mice, whereas the Armstrong strain virus is not. These findings indicate a relative inefficiency of the reticuloendothelial cells in the phagocytosis of virus particles of the Armstrong strain, and this could account for the relative virulence of the WE₃ strain virus on extraneural inoculation. Roberts (1964) was able to associate the virulence of a strain of ectromelia virus with its ability to infect mouse peritoneal macrophages and Kupffer cells in the liver much more readily than an attenuated strain of the virus.

When LCM virus is injected intraperitoneally in mice, it first encounters the peritoneal macrophages. Since few of these cells become infected with the Armstrong strain virus, this would account for the failure of development of significant local and visceral infection in this case. After intravenous injection of the WE₃ strain virus, most of the virus is taken up from the blood by Kupffer cells lining the liver sinusoids and to a lesser extent by macrophages in the spleen (see Section IV). It was shown that hepatic cells are not infected directly, but only after multiplication of virus has occurred in the Kupffer cells. Lack of uptake of Armstrong strain virus by the Kupffer cells would explain the failure of significant infection to develop in the
viscera after intravenous injection of this strain of virus. When virus is injected intracerebrally, it enters the CSF spaces as well as spilling over into the blood (Cairns, 1950; Mims, 1960a). After intracerebral injection of LCM virus, both $WE_3$ and Armstrong strains of virus were able to grow in the meninges, ependyma and choroid plexus epithelium, apparently independent of phagocytosis by cells of the reticuloendothelial system. The reason for the lesser growth of the Armstrong strain virus in the CNS (and also in the foot after footpad infection) is not clear, but a more vigorous immune response to infection with this strain of virus may have played a part.

In infant mice, it was found that $WE_3$ strain virus was not cleared rapidly from the blood as it was in adult mice, and neither $WE_3$ nor Armstrong strain virus was detectably cleared by sixty minutes after intravenous injection. Nevertheless, both strains of virus caused extensive visceral infection in infant mice. One possible explanation would be that clearance takes longer to occur in infant mice, and that even fifty per cent of each strain of virus could in fact have been cleared by sixty minutes without being detected (as a $10^{0.3}$ fall in blood titre) by the rather insensitive assay method. It has been reported that newborn mice fail to clear
Sindbis virus from the blood as effectively as adults (Johnson & Mims, 1968). Another possibility is that in infant mice, LCM virus is able to infect parenchymal cells of the visceral organs, such as hepatic cells of the liver, directly from the blood. In adult mice, virus is taken up from the blood by the Kupffer cells and it is only after replication in these cells that hepatic cells are infected (see Section IV). If direct infection of parenchymal cells could occur in infant mice, this would enable both the WE₃ and Armstrong strains of virus to cause infection of the viscera, regardless of the phagocytic capacity of the reticuloendothelial cells. The lack of rapid clearance of either strain of virus could also account for the ability of both strains to enter the CNS of infant mice from the blood at an early stage (see Section V), since viraemia is maintained after injection.

It was found that carrier virus behaved like the Armstrong strain of virus, when injected in adult mice, indicating that it had become mouse-adapted by its passage through many generations of mice. Unlike WE₃ strain virus, carrier virus and Armstrong strain virus were not rapidly cleared from the blood after intravenous injection. It is possible that during growth
in mouse tissues, LCM virus develops some antigenic similarity to mouse cells and is subsequently not recognized as "foreign" by cells of the reticuloendothelial system. Since LCM virus particles mature by budding from the cell membrane and have a lipoprotein envelope and surface projections (Dalton et al., 1968; Compans, R.W., unpublished data), the virus particles might incorporate host antigen, as occurs with influenza virus (Laver & Webster, 1966). It has been reported that although LCM virus passaged intracerebrally in mice caused a high mortality when injected into newborn mice, the mortality was markedly reduced by repeated passage of the virus intraperitoneally in mice (Hotchin et al., 1962), but the mechanism of this change is not clear. Although a large number of mouse passages may be required to convert a strain of LCM virus to the fully mouse-adapted state and the transition appears to be a gradual one (Schwartzman, 1946), the acquisition of some characteristics of a mouse-adapted strain of virus may occur rapidly. When pregnant mice are infected with WE3 strain virus, infection of the foetus occurs (Mims, 1969 b) and the pregnancy can be maintained until term by treatment with antilymphocyte serum. Mims (unpublished data) found that when virus was obtained from infected foetal or neonatal mice and
injected intracerebrally in adult mice, some deaths occurred earlier than with WE₃ strain virus and the median survival time was similar to that following Armstrong strain virus infection.

SUMMARY

The response of mice to infection with the WE₃ and Armstrong strains of LCM virus has been investigated. The effects of injection of virus by various routes were determined, in adult and in infant mice. After intravenous or intraperitoneal injection, the WE₃ strain virus readily caused infection of the viscera and was able to enter the CNS, both in infant and in adult mice. The Armstrong strain virus caused extensive infection in the viscera and the CNS of infant mice, but in adult mice significant infection did not occur. After intracerebral injection, however, both strains of virus caused CNS infection in adult mice as well as in infant mice. When virus was injected into the footpad, local growth of both strains of virus occurred and the primary footpad swelling was compared. A comparison was also made of the hypersensitive footpad response to footpad challenge, in mice previously sensitized by infection with either WE₃ or Armstrong strain virus. Evidence was obtained that Armstrong strain
virus infection gives rise to a more intense immune response than WE₃ strain virus infection. This difference in immune response was thought to account for some of the differences in behaviour of the two strains of virus in mice.

The WE₃ strain virus was rapidly cleared from the blood of adult mice, but the Armstrong strain virus was not. In infant mice, neither strain of virus was cleared rapidly after intravenous injection. It was found that mouse peritoneal macrophages were more readily infected by WE₃ strain virus than by Armstrong strain virus. The possible relationships between these observations and the results of infection of adult and infant mice by various routes have been discussed.

Carrier virus was found to behave in adult mice like the Armstrong strain, mouse-adapted virus.
XI. THE RESPONSE OF MICE OF DIFFERENT STRAINS TO INFECTION WITH LYMPHOCYTIC CHORIOMENINGITIS VIRUS

It has been found that different strains of mice show differences in susceptibility to contact infection (Traub, 1936 b) and to intracerebral injection (Hotchin & Weigand, 1961 a; Oldstone & Dixon, 1968) with LCM virus. Also, LCM virus carrier mice of different strains show different titres of the virus in their organs (Volkert & Hannover Larsen, 1965 a; Oldstone & Dixon, 1968, 1969). An investigation was therefore made of the mechanisms by which different strains of mice show different responses to infection with LCM virus. In particular, mice of the C₅₇BL strain were compared with WEHI mice, because there is evidence that C₅₇BL mice show a more vigorous immune response to infection with ectromelia virus (Schell, 1960 a) and it is the immune response to LCM virus infection that is thought to cause disease and death (Rowe, 1954; Hotchin, 1962 a).

RESULTS

A comparison was made of the response of different strains of mice to infection by various routes with LCM virus, with regard to mortality, growth of virus in the tissues and the development of pathological changes.
<table>
<thead>
<tr>
<th>STRAIN OF MICE</th>
<th>MORTALITY AFTER INTRACEREBRAL OR INTRAPERITONEAL INFECTION</th>
<th>PATHOLOGICAL CHANGES IN LIVER AND SLEEN AFTER INTRACEREBRAL OR INTRAPERITONEAL INFECTION</th>
<th>PLEURAL AND PERITONEAL EFFUSIONS AFTER INTRAPERITONEAL INFECTION</th>
<th>HYPERSENSITIVE FOOTPAD SWELLING</th>
</tr>
</thead>
<tbody>
<tr>
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<td>+ + + +</td>
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<td>C3H</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
</tbody>
</table>

**Table 21.**

EFFECTS OF LCM VIRUS INFECTION IN DIFFERENT STRAINS OF MICE.
A. Response to Intracerebral Infection with LCM Virus.

Mortality

The mortality after intracerebral injection was compared in mice of the WEHI, C_{57}BL, Bagg and C_{3}H strains. Groups of six mice of each strain were injected intracerebrally with tenfold dilutions of a guinea pig lung suspension of WE_{3} strain LCM virus, from $10^{-1}$ to $10^{-8}$. Although occasional WEHI mice survived the injection of very large doses of virus, the deaths of these mice showed a well defined end-point, at the $10^{-7}$ dilution. However, Bagg, C_{3}H and especially C_{57}BL mice were much more resistant to the lethal effects of intracerebral injection of LCM virus. Some mice usually survived and recovered at all dilutions and in the mice that died, the mean survival time was generally several days longer than in the case of WEHI mice. The comparative mortality of the different strains of mice is shown on an arbitrary scale in Table 21.

Growth of Virus in the Tissues

Groups of six WEHI, C_{57}BL, Bagg and C_{3}H mice were injected intracerebrally with $10^{5}LD_{50}$ of WE_{3} strain LCM virus and at five days, frozen sections of the brains, livers and spleens were stained with fluorescein-conjugated antiserum against LCM virus. In the WEHI mice, infection
Table 22. VIRUS TITRES IN BRAIN AND SPLEEN OF DIFFERENT STRAINS OF MICE AT FIVE DAYS AFTER INTRACEREBRAL INJECTION OF $10^5\text{LD}_{50}$ OF WE3 STRAIN LCM VIRUS.

<table>
<thead>
<tr>
<th>STRAIN OF MICE</th>
<th>VIRUS TITRE ($\log_{10}\text{LD}_{50}$ per gram)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BRAIN</td>
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<td>WEHI</td>
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<tr>
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</table>
was present in the meninges, choroid plexuses, ependyma and Virchow-Robin spaces and there was no extension into the brain substance, apart from some infected cells in submeningeal and subependymal areas. The liver showed widespread infection of Kupffer cells and hepatic cells and in the spleen there was marked infection, mainly in perifollicular regions and in the red pulp. The other strains of mice showed no detectable difference in the pattern of infection in the CNS, the liver or the spleen.

At five days, suspensions of the brains and spleens were titrated intracerebrally in WEHI mice and the titres are shown in Table 22. It can be seen that there were no significant differences in brain virus titres in the four strains of mice. In the spleens, the virus titres were lower in WEHI mice than in the C₅₇BL, Bagg or C₃H mice.

Development of Pathological Changes in the Tissues

Groups of six mice of the WEHI, C₅₇BL, Bagg, C₃H and also of the CBA and A strains were injected intracerebrally with 10⁵LD₅₀ of WE₃ strain LCM virus and at six days, the brains, livers and spleens were examined by routine histology. In WEHI mice, there was a marked mononuclear cell infiltration in the meninges,
choroid plexuses and some Virchow-Robin spaces. The infiltrating cells were mostly large mononuclears, with a small number of small lymphocytes and few if any plasma cells and polymorphonuclear leukocytes. The liver showed a marked mononuclear cell infiltration in the portal tracts and sinusoids, with numerous small foci of mononuclear cells in the sinusoids. There were scattered necrotic hepatic cells, singly or in small groups, surrounded by collections of mononuclear cells. The cells infiltrating the liver were mostly large mononuclears, with small numbers of small lymphocytes, plasma cells and polymorphonuclear leukocytes. A marked degree of fine vacuolation was present in the hepatic cells and by fetrot staining of formalin-fixed frozen sections, this was shown to be fatty infiltration. In the spleen, there were many large pale mononuclear cells around the follicles. Widespread fibrinoid necrosis had occurred, mainly in perifollicular regions, but extending as a loose network into some follicles, which then contained only a few lymphoid cells. The development of these histological changes is described in detail in Sections IV, V and VI.

In the $C_{57}$BL, Bagg, $C_{3}$H, CBA and A strain mice, the lesions were similar in type to those in the WEHI mice,
but lesser in frequency and severity. Although a mononuclear cell infiltration was present in the liver, necrotic hepatic cells were much less numerous and were mostly single. In the spleen, there were many large pale mononuclear cells around the follicles, but the fibrinoid necrotic lesions were much less marked than in the WEHI mice and were confined to a few perifollicular areas. A comparison of the pathological changes in the different strains of mice is made in Table 21. Thus, the pathological changes which occurred in the visceral organs after LCM virus infection were most severe in the WEHI mice. The splenic lesions were greatest in this strain, although the virus grew to higher titres in the spleen in the three other strains of mice tested (Table 22). The most important factor in determining the severity of the pathological changes in different strains of mice, therefore, does not appear to be the level of virus growth in the tissues.

It was thought possible that WEHI mice carry an infectious agent which enhances the production of lesions in LCM virus infection. Groups of male and female C57BL mice were therefore caged for three months with groups of WEHI mice of the same sex. The mice were then injected intracerebrally with $10^5 \text{LD}_{50}$ of WE3 strain.
LCM virus and at six days, the liver and spleen were examined histologically. The lesions were still much less severe in the C\textsubscript{57}BL mice than in the WEHI mice. There was therefore no indication of the transmission of an infectious agent by contact with the WEHI mice. To test for the possible intrauterine (vertical) transmission of an infectious agent, WEHI and C\textsubscript{57}BL mice were crossed, using both males and females of each strain as parents. When the F\textsubscript{1} offspring were six weeks old, they were injected intracerebrally with $10^5LD_{50}$ of WE\textsubscript{3} strain LCM virus. At six days, histological examination of the liver and spleen showed that all the F\textsubscript{1} mice developed lesions of the severity seen in WEHI mice. Thus, the development of severe pathological changes in WEHI mice infected with LCM virus is most probably due to a greater genetic susceptibility.

B. Response to Intraperitoneal Infection with LCM Virus. Pleural and Peritoneal Effusions and Mortality

In systemic LCM virus infection in mice, the exudation which occurs into the pleural and peritoneal cavities appears to be due to the immune response to the virus, since this exudation is inhibited by X-irradiation (Rowe, 1954, 1956). Groups of eighteen mice of the WEHI, C\textsubscript{57}BL, Bagg and C\textsubscript{3}H strains were
<table>
<thead>
<tr>
<th>STRAIN OF MICE</th>
<th>VOLUME OF PLEURAL FLUID (mls.)</th>
<th>VOLUME OF PERITONEAL FLUID (mls.)</th>
<th>MORTALITY [Number of Deaths/Number of mice infected]</th>
<th>MEDIAN SURVIVAL TIME (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WEHI</td>
<td>1.58</td>
<td>0.66</td>
<td>12/12</td>
<td>7.5</td>
</tr>
<tr>
<td>C₅₇BL</td>
<td>0.09</td>
<td>0.14</td>
<td>0/12</td>
<td>-</td>
</tr>
<tr>
<td>BAGG</td>
<td>0.21</td>
<td>0.40</td>
<td>6/12</td>
<td>9.7</td>
</tr>
<tr>
<td>C₃H</td>
<td>0.14</td>
<td>0.22</td>
<td>6/12</td>
<td>15.5</td>
</tr>
</tbody>
</table>

**Table 23.** EXUDATION INTO BODY CAVITIES AT EIGHT DAYS AND MORTALITY IN DIFFERENT STRAINS OF MICE AFTER INTRAPERITONEAL INJECTION OF $10^5LD_{50}$ OF WE₃ STRAIN LCM VIRUS.
injected intraperitoneally with $10^5 \text{LD}_{50}$ of WE$_3$ strain LCM virus. At eight days, six mice from each group were killed, as much as possible of the pleural and peritoneal fluid aspirated and the volumes measured. The mean volumes are shown in Table 23, together with mortality among the remaining mice, and a comparison between the different strains of mice is made in Table 21. It can be seen that the WEHI mice, which showed the highest mortality, had the largest pleural and peritoneal effusions and C$_{57}$BL mice, with the lowest mortality, had the smallest effusions. The other two strains were intermediate, both in mortality and in volume of effusions. Histological Examination

At eight days, histological examination of the liver and spleen of the WEHI mice showed a marked mononuclear cell infiltration and widespread necrotic changes, similar to those following intracerebral infection (see page 182). In the other strains of mice, the lesions were again much less marked, though similar in type to those in WEHI mice, and a comparison is made in Table 21. There is a correlation between the mortality, the effusions and the pathological changes in the different strains of mice, and these are probably all indications of the magnitude of the immune response to LCM virus.
Figure 56. Primary footpad swelling in different strains of mice after footpad injection of $10^5 LD_{50}$ of WE$_3$ strain LCM virus.
<table>
<thead>
<tr>
<th>STRAIN OF MICE</th>
<th>PRIMARY FOOTPAD SWELLING</th>
<th>PATHOLOGICAL CHANGES IN POPLITEAL LYMPH NODE</th>
<th>OBSTRUCTION TO LYMPHATIC DRAINAGE OF FOOT</th>
</tr>
</thead>
<tbody>
<tr>
<td>WEHI</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>C₅₇BL</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>BAGG</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>C₃H</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Table 24.** Effects of footpad injection of $10^5 \text{LD}_{50}$ of WE₃ strain LCM virus in different strains of mice.

**Figure 96.** Primary footpad swelling in different strains of mice after footpad injection of $10^5 \text{LD}_{50}$ of WE₃ strain LCM virus.
C. Response to the Footpad Injection of LCM Virus.

Primary Footpad Swelling

Primary footpad swelling in mice after footpad infection with LCM virus is probably due to a combination of the humoral and cellular immune response to the virus (see Section IX). The primary footpad swelling was compared in mice of the WEHI, C57BL, Bagg and C3H strains. Groups of twelve mice of each strain were injected subcutaneously in a hind footpad with 10^5 LD_{50} of WE3 strain LCM virus. The feet were measured at daily intervals and the mean percentage increase in footpad thickness is charted in Figure 56, with a comparison of the different strains in Table 24. The WEHI mice showed the greatest, and the C3H mice the least, degree of footpad swelling. Five of the twelve Bagg mice died, with a mean survival time of twelve days, and the mice which died showed less footpad swelling than those which survived. No other mice died.

Immunofluorescence and Histological Examination

At nine days, the brain, liver and spleen were removed for immunofluorescence examination and the brain, liver, spleen and popliteal lymph node were examined by routine histology. In the Bagg mice, there was infection of the meninges, choroid plexuses, ependyma and cerebral
blood vessels, but no infection was detected in the CNS of the WEHI, C₅₇BL and C₃H mice. Histological examination in Bagg mice showed a mild to moderate mononuclear cell infiltration in the meninges, choroid plexuses and Virchow-Robin spaces, but these changes were not present in the other strains of mice. Infection in the liver and spleen was most marked in the Bagg and WEHI mice and least in the C₅₇BL and C₃H mice, but there was no difference in the pattern of infection. In the liver, there was infection of hepatic cells and Kupffer cells and in the spleen, infected cells were present mainly in the perifollicular regions and the red pulp. Histological examination in WEHI mice showed the following changes. In the liver, a marked mononuclear cell infiltration was present in the portal tracts and sinusoids, with numerous small foci of mononuclear cells in the sinusoids. There were scattered necrotic hepatic cells, singly or in small groups, surrounded by collections of mononuclear cells. In the spleen, many large pale mononuclear cells were present around the follicles. Widespread fibrinoid necrosis had occurred, mainly in perifollicular regions, but extending into the centre of some follicles. The popliteal lymph node showed marked oedema and lymphoid cell depletion. Widespread fibrinoid
necrosis had occurred in the node and scattered nuclear debris was present. In the C57BL, Bagg and C3H mice, there was a marked mononuclear cell infiltration in the liver, but few necrotic hepatic cells were seen. The spleen showed perifollicular collections of large pale mononuclear cells, but only small amounts of fibrinoid necrosis had occurred, in perifollicular regions. In the popliteal lymph node, oedema and lymphoid cell depletion were less marked than in the WEHI mice and only small amounts of fibrinoid necrosis had occurred, mainly at the periphery of the node. A comparison between the different strains is made in Table 24.

Thus, at nine days after footpad injection of LCM virus, infection of the viscera was most marked in WEHI and Bagg mice and CNS infection had developed only in Bagg mice. Necrotic changes in the liver, spleen and popliteal lymph node were most severe in WEHI mice.

Growth of Virus in the Foot

In view of the marked difference in primary footpad swelling, the growth of LCM virus in the inoculated feet was compared in the different strains of mice. Groups of six mice of each strain were injected subcutaneously in the footpad with $10^2 LD_{50}$ of WE3 strain LCM virus and at six days, the day before footpad swelling
<table>
<thead>
<tr>
<th>STRAIN OF MICE</th>
<th>VIRUS TITRE IN INJECTED FOOT (log_{10}LD_{50} per gram)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WEHI</td>
<td>5.2</td>
</tr>
<tr>
<td>C_{57}BL</td>
<td>5.2</td>
</tr>
<tr>
<td>BAGG</td>
<td>5.1</td>
</tr>
<tr>
<td>C_{3}H</td>
<td>5.3</td>
</tr>
</tbody>
</table>

Table 25. VIRUS TITRES IN FEET OF DIFFERENT STRAINS OF MICE AT SIX DAYS AFTER FOOTPAD INJECTION OF 10^{-2}LD_{50} OF WE_{3} STRAIN LCM VIRUS.
would have occurred with this dose of virus (see Section IX), the feet were removed and titrated. The results are shown in Table 25, and it can be seen that virus titres in the feet of the WEHI, C\textsubscript{57}BL, Bagg and C\textsubscript{3}H mice were identical.

**Impedance of Lymphatic Drainage**

It has been shown that one factor which contributes to primary footpad swelling after footpad injection of LCM virus is the development of obstruction to the lymphatic drainage of the foot (see Section IX). The degree of obstruction was found to vary with the strain of LCM virus injected into the footpad (see Section X). An experiment was done to determine whether there was any variation in the degree of lymphatic obstruction which developed in different strains of mice. Groups of six mice of the WEHI, C\textsubscript{57}BL, Bagg and C\textsubscript{3}H strains were injected subcutaneously in the footpad with 10\textsuperscript{5}LD\textsubscript{50} of WE\textsubscript{3} strain virus. At eight days, when there was marked footpad swelling, 0.03 ml of India ink was injected into the infected footpad and into the footpad of normal WEHI mice as controls. Twenty-four hours later, the popliteal lymph node was removed and examined histologically. In the normal WEHI lymph nodes, there were large amounts of India ink in the afferent lymphatics and marginal sinus,
extending into the medullary cords. The infected WEHI lymph nodes, on the other hand, showed small amounts of India ink in the afferent lymphatics and only a few particles in the nodes themselves, in the marginal sinus. There were small amounts of India ink in the afferent lymphatics and marginal sinus in the C57BL mice, moderate amounts in the Bagg mice and rather larger amounts in the C3H mice. A comparison between the different strains is made in Table 24. Thus, in all the strains of mice there was impedance of the lymphatic drainage of the foot and it can be seen that there was a correlation between the degree of lymphatic obstruction, the severity of pathological changes in the popliteal lymph node and the degree of primary footpad swelling observed in each strain of mice (Table 24). Since the oedema and necrotic changes in the popliteal lymph node appear to result from the immune response to LCM virus infection (see Section VI), the degree of lymphatic obstruction may reflect the magnitude of the immune response in the different strains of mice, as well as contributing to the footpad swelling.

Hypersensitive Footpad Swelling

In the hypersensitive footpad reaction to LCM virus, the early (four hour) swelling probably depends on the humoral immune response and the delayed (twenty-four
### Table 26.

**Hypersensitive Footpad Swelling in Different Strains of Mice Following Footpad Challenge with $10^7 \text{LD}_{50}$ of WE$_3$ Strain LCM Virus at Ten Days after Intraperitoneal Injection of $10^4 \text{LD}_{50}$ of Armstrong Strain Virus.**

<table>
<thead>
<tr>
<th>STRAIN OF MICE</th>
<th>MEAN PERCENTAGE INCREASE IN FOOTPAD THICKNESS - TIME AFTER FOOTPAD INJECTION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 hours</td>
</tr>
<tr>
<td>WEHI</td>
<td>32.3 ± 10.8*</td>
</tr>
<tr>
<td>C$_{57}$BL</td>
<td>39.4 ± 12.3</td>
</tr>
<tr>
<td>BAGG</td>
<td>33.7 ± 9.6</td>
</tr>
<tr>
<td>C$_3$H</td>
<td>34.7 ± 4.2</td>
</tr>
</tbody>
</table>

*Standard Deviation
to forty-eight hour) swelling depends on the cellular hypersensitivity response (see Section IX). The following experiment was done to compare hypersensitive footpad swelling in mice of the WEHI, C\textsubscript{57}BL, Bagg and C\textsubscript{3}H strains. Groups of fourteen mice of each strain were infected intraperitoneally with $10^4$LD\textsubscript{50} of Armstrong strain LCM virus and ten days later, a dose of $10^7$LD\textsubscript{50} of WE\textsubscript{3} strain virus was injected subcutaneously into the footpad. The feet were measured at four, twenty-four and forty-eight hours and the mean percentage increases in footpad thickness are shown in Table 26. It can be seen that the four-hour phase of the hypersensitive footpad swelling was not significantly different in the four strains of mice. The delayed footpad swelling was greatest in the Bagg mice and least in the C\textsubscript{3}H mice, and a comparison is made in Table 21. In so far as the delayed swelling reflects the cellular hypersensitivity response to LCM virus, this response is most intense in the Bagg mice, and it is more marked in the WEHI mice than in the C\textsubscript{57}BL mice and least of all in the C\textsubscript{3}H mice.

**DISCUSSION**

The importance of genetic influences in susceptibility to viral infections is well known (Gowen, 1948; Smorodintsev, 1960; Bang & Luttrell, 1961).
The basis of variation of susceptibility may depend on the nature of the particular infection. In mouse hepatitis virus infection, strain variation in susceptibility seems to be resident in the cells, because macrophages from a susceptible strain of mice were shown to be more susceptible to infection in vitro, whereas macrophages from a resistant strain of mice were resistant (Bang & Warwick, 1960). There is also evidence for differences in cell susceptibility to infection with St. Louis encephalitis, yellow fever and antigenically related viruses (Webster & Johnson, 1941; Sabin, 1954). In the case of ectromelia virus, however, Roberts (1964) found that macrophages from susceptible and resistant strains of mice were equally susceptible to infection in vitro. In ectromelia virus infection, in which the immune response is protective, Schell (1960 a) produced evidence that C57BL mice showed a more intense immune response than WEHI mice, which were more susceptible to the infection. In mice infected with LCM virus, the immune response appears to be the cause of disease and death (Rowe, 1954; Hotchin, 1962 a), so the susceptibility of different strains of mice to LCM virus infection may depend on the intensity of their immune response to the virus. In different strains of mice, differences have been demonstrated in the antibody response (Davidsohn & Stern, 1949; Fink & Quinn, 1953; Ipsen, 1954, 1959) and
also in the delayed type hypersensitivity response (Crowle, 1959 b) and the graft versus host reaction (Gorer & Boyse, 1959), both of which involve cellular hypersensitivity.

Different strains of mice showed differences in susceptibility to intracerebral infection with LCM virus, even though the virus grew to identical titres in the brains (Table 22). Virus titres in the spleen were lowest in WEHI mice, although these mice showed the most marked pathological changes in the spleen and liver and the highest mortality. These observations suggest that there is a variation in the magnitude of the immune response to LCM virus in the different strains of mice. The immune response would appear to be most intense in the WEHI mice, which showed the greatest susceptibility to infection, and least intense in the C₅⁷BL mice, which were least susceptible. Mims, C.A. (unpublished data) found that the levels of neutralizing antibodies in the serum at six days after intracerebral infection were the same in C₅⁷BL mice as in WEHI mice, so the cellular immune response assumes a greater importance. Differences in the susceptibility of adult mice of different strains to intracerebral infection with LCM virus have also been demonstrated by Oldstone & Dixon (1968), and there appeared to be a correlation with the response of neonatal
mice of the different strains to infection with the virus (Oldstone & Dixon, 1969). Although C₅₇BL mice give a more vigorous immune response to ectromelia virus than do WEHI mice (Schell, 1960 a), they appear to give a weaker (cellular) immune response to LCM virus. The fact that C₅₇BL mice are more resistant to both ectromelia and LCM virus infections illustrates that the immune response may have a beneficial or an adverse effect, depending on the infectious agent.

After footpad infection with LCM virus, the same amount of virus growth occurred in the foot in different strains of mice (Table 25), but there was a marked variation in the amount of primary footpad swelling (Figure 56), again suggesting a variation in the immune response. There was a correlation between the amount of footpad swelling and the severity of the pathological changes in the popliteal lymph node (Table 24), which are probably due to the immune response to the infection (see Section VI). After intraperitoneal infection of different strains of mice with LCM virus, there was a correlation between mortality, pathological changes in the visceral organs and the volume of pleural and peritoneal effusions (Table 21). These effects are all indications of the intensity of the immune response (Section VI; Rowe, 1954; Hotchin, 1962 a) and were
greatest in WEHI mice and least in C$_{57}$BL and C$_3$H mice. In the hypersensitive footpad response to LCM virus in sensitized mice, the early phase of the swelling was not significantly different in all the strains of mice tested, and this may indicate a similar humoral antibody response (see Section IX). The delayed swelling was less marked in C$_3$H and C$_{57}$BL mice than in WEHI mice, and this reflects a less vigorous cellular immune response of C$_3$H and C$_{57}$BL mice to LCM virus infection, which is consistent with and helps to account for the phenomena summarized in Table 21.

In considering all the manifestations of the immune response to LCM virus (Tables 21, 24), it is apparent that the responsiveness of different strains of mice varies according to the route of injection of the virus. Schell (1960 b) found that strain differences in susceptibility to ectromelia virus were most pronounced when the virus was injected in the footpad or intravenously, but the differences were abolished when the intracerebral or intraperitoneal routes of injection were used. In particular, Bagg mice show some irregularities in their response to LCM virus infection as correlated with the immune response. For instance, their very marked hypersensitive footpad swelling is unexpected, in view of the relative resistance of these mice to intracerebral or intraperitoneal infection.
with LCM virus. It has been found that the delayed phase of the hypersensitive footpad response to polymerized flagellin is more marked in Bagg mice than in mice of other strains (Ada, G.L., unpublished data). To explain the resistance of some mice to high doses of LCM virus, it has been suggested that high doses may induce immunological tolerance or paralysis in adult mice (Hotchin & Benson, 1963). In newborn mice, Lehmann-Grube (1964 b) has shown that immunological tolerance to LCM virus is induced in a much higher proportion of mice by infection with a very large dose of virus than with a small dose. It is possible that the ease with which immunological tolerance may be induced varies in different strains of mice (Sobey & Magrath, 1965). When WEHI mice were infected neonatally with a large dose of WE3 strain LCM virus, thirty per cent of the mice died (Lehmann-Grube, 1964 b), but in C57BL mice, similar infection caused zero mortality (Mims, C.A., unpublished data). Perhaps Bagg mice have a greater capability for developing a vigorous cellular immune response to LCM virus, but this is readily suppressed by high doses of virus. This might account for the relative resistance of Bagg mice to intracerebral or intraperitoneal infection with LCM virus, whereas their marked susceptibility to the lethal effects of footpad infection may be due to a gradual release of virus from the footpad into the circulation.
After primary footpad infection with LCM virus, it was found that different strains of mice differ in their ability to limit the spread of virus from the foot. Footpad injection of $10^5 \text{LD}_{50}$ of WE$_3$ strain virus in Bagg mice caused approximately forty per cent mortality, whereas WEHI mice remained healthy after this dose of virus. However, if a larger dose ($10^7 \text{LD}_{50}$) is injected into the footpad of WEHI mice, some deaths do occur (see Section IX). Variations in mortality after footpad inoculation of LCM virus in mice of different strains has also been found by Roger & Roger (1963 a, b). The progress of the primary footpad response in Bagg mice (Figure 56) suggests that when the mice become sick, footpad swelling fails to increase or even diminishes, but if they do not die, increase in footpad swelling may occur as they recover. This sequence of events may reflect the local (footpad swelling) and the systemic (sickness and death) manifestations of the immune response, since LCM virus was shown to spread from the footpad to the viscera and the brain in Bagg mice. Probably the immune response, although it eventually causes the lesions, also plays a part in limiting the extent of the infection and hence the extent of the lesions. The disease picture reflects the balance between the infection-limiting and the lesion-producing activities of the immune
response. Mims (1969 b) found that although potent antilymphocyte serum protected mice from the lethal effects of intravenous LCM virus infection, treatment with weak antilymphocyte serum caused an increased susceptibility to the infection. Splenectomy also resulted in an increased susceptibility of mice to intravenous LCM virus infection (Mims, C.A., unpublished data).

SUMMARY

The response of mice of different strains to infection by various routes with LCM virus has been compared. In general, WEHI mice showed the most severe, and C₅₇BL and C₃H mice the least severe, reaction to LCM virus infection.

WEHI mice were most susceptible to the lethal effects of intracerebral infection, even though brain virus titres were the same as in the other strains of mice. Primary footpad swelling after footpad infection was greatest in WEHI mice, and virus growth in the foot was the same in all the strains of mice tested. However, pathological changes in the local lymph node and obstruction of the lymphatic drainage of the foot were greatest in the WEHI mice. After intraperitoneal infection, mortality was greatest in WEHI mice and least in C₅₇BL mice and this correlated with the severity of the pathological changes
in the liver and spleen and the volume of pleural and peritoneal effusions. Hypersensitive footpad swelling, as a measure of the cellular immune response, was greater in WEHI than in C57BL mice. The importance of the immune response of the different strains of mice in determining their susceptibility or resistance to LCM virus infection has been discussed.
In many infections harmful to animals or man, the predominating factor in the pathogenesis of the disease is the direct cytopathic effect of the microorganism. However, in infection with an agent which is basically non-cytopathic, such as LCM virus, the mechanisms causing disease are more difficult to evaluate. A knowledge of the evolution of the infection from the earliest stages to the final lesions is essential for a complete understanding of the pathogenesis of the disease.

The natural route of infection of the visceral organs and of the CNS is via the blood stream. The immunofluorescence technique has enabled the study of the entry of LCM virus from the blood into the liver and spleen and into the CNS of mice. The initiation and spread of the infection could be accurately followed, and the relation between the sites of infection and the appearance of pathological changes in the tissues has been studied. It has been shown that infection of the phagocytic cells of the reticuloendothelial system is of basic importance in the initiation of infection in the liver and spleen. Virus replicated in the Kupffer cells of the liver before infection was passed on to hepatic cells. Macrophages were infected less readily with
mouse-adapted strains of LCM virus and extraneural inoculation did not result in significant infection of the viscera. The importance of macrophages in many other viral infections has been stressed by Mims (1964 a).

The pathological lesions in the lymphoid tissues and liver of mice infected with LCM virus appeared within foci of infected cells. The lesions were shown to be fibrinoid in nature and were apparently due to an interaction between infected cells and sensitized lymphoid cells. The ability of LCM virus to infect lymphoid cells without preventing their immunological activities may be important in the pathogenesis of the infection. In mice infected with LCM virus, depression of the immune response to unrelated antigens occurs (Mims & Wainwright, 1968) and this appears to require infection of lymphoid cells, accompanied by a significant immune response to the infection. Immunodepression also occurs in infections with murine leukaemia viruses (Dent et al., 1965; Salaman & Wedderburn, 1966; Siegel & Morton, 1966), murine cytomegalovirus (Osborn et al., 1968) and Junin (Parodi et al., 1967) and Aleutian disease (Kenyon, 1966) viruses, in all of which the lymphoid tissues are involved. Non-cytopathic infection of lymphoid cells may prove to be of paramount importance in the pathogenesis of these persistent viral infections.
Cell-mediated immune responses may be beneficial or harmful in different infections, depending on the nature of the micro-organisms involved. They are important in acquired resistance to facultative intracellular parasites, such as Listeria, Brucella and Salmonella organisms (Mackaness, 1967) and in resistance to virus-induced tumours (Allison, 1967). On the other hand, the cellular immune response may play an important part in the pathogenesis of lesions in a number of conditions, including infections with rickettsiae, poxviruses and measles virus (Mims, 1966 b; Allison, 1967). In LCM virus infection, the results of the present studies are consistent with the many different lines of evidence which incriminate the cellular immune response as the principal factor causing disease and death. However, the actual effect in vivo of an interaction between a sensitized lymphoid cell and an infected cell still remains unknown. In vitro, the tissue culture methods developed by Rosenau & Moon (1961) have been applied to LCM virus infection by Lundstedt (1969), who obtained some evidence for a cytocidal interaction between sensitized lymphoid cells and cells infected with LCM virus. Further investigations along these lines may help to establish the mechanism of production of pathological changes in the infected animal. The graft rejection experiments of
Holtermann & Majde (1969) have indicated the possibility of an antigenic difference between cells infected with LCM virus and uninfected cells, and the extension of these experiments may elucidate the nature of this difference. A major problem is to obtain from acutely infected mice, or from carrier mice, lymphoid and other cells which are free of infectious LCM virus.

Although the cellular immune response is of prime importance in the pathogenesis of LCM virus infection, the possible participation of antibodies should not be overlooked. The presence of immunoglobulin in the pathological lesions in the lymphoid tissues and liver suggests that antibodies may play a part in the development of the lesions. Antiviral antibody may adsorb to the surface of infected cells, since LCM virus particles mature at the cell surface (Dalton et al., 1968), and this might result in damage to the cells. It has been shown that anticellular antibody can cause lysis of the cells to which it is adsorbed (Dumonde et al., 1965), and antiserum to rabies virus may cause lysis of tissue culture cells infected with the virus (Wiktor et al., 1968). Also, the deposition of circulating virus-antibody complexes at sites of early tissue damage may accelerate this damage, as in Auer hepatitis (Paronetto & Popper, 1965). It is possible that antibody against LCM virus participates in the response
to CNS infection, in primary and hypersensitive footpad swelling and in the production of pleural and peritoneal effusions. The question of antibody involvement might be further elucidated by use of the recently-developed immunofluorescence method of determination of antibody levels (Benson & Hotchin, 1969), which is much more sensitive than tests for complement fixing or neutralizing antibodies. A haemagglutination-inhibition test for antibodies to LCM virus may yet be developed.

The haematogenous spread of LCM virus into the CSF is an important mode of initiation of meningitis and choroiditis. It has been found that the earliest route of entry into the CSF was through the blood vessels of the meninges, and passage of virus through the choroid plexuses appeared to be of subsidiary importance. Infection of cerebral blood vessels occurred, but only in infant mice did the infection extend into the brain substance. The failure of infection of the brain substance in adult mice, even after direct intracerebral injection of LCM virus, is an important difference from viruses causing encephalitis, which are able to enter the brain substance from the CSF or the cerebral blood vessels (Johnson & Mims, 1968).

The adoptive immunization of LCM virus carrier mice was shown to result in the production of complement fixing and neutralizing antibodies, in confirmation of
the work of Volkert (1965). However, although circulating free virus was neutralized, there was no reduction in viral antigen in the tissues, as detected by immunofluorescence, and no pathological changes occurred. That is, immunological tolerance to LCM virus was readily reversed in so far as antibody production was concerned, but the capacity for a cellular immune response could not be conferred. This result is unexpected, in view of the successful transfer of transplantation immunity by the adoptive immunization of immunologically tolerant animals (Billingham et al., 1956; Gowans, 1965). Perhaps the most likely explanation is that the transferred lymphoid cells were desensitized by contact with LCM virus antigen, which is widespread in carrier mice. As a result of the persistent infection, LCM virus carrier mice may develop certain chronic diseases (Hotchin, 1962 a, 1965; Pollard et al., 1968 a, b; Mims, 1969 c). Immunological tolerance may be important in persistent infections with other viruses, such as avian leukosis (Burmester, 1962; Rubin et al., 1962), mammary tumour and murine leukaemia (Old & Boyse, 1965; Klein, 1968) viruses. There are a number of points of similarity between LCM virus and murine leukaemia viruses (Traub, 1960 a; Mims, 1968 b),
and it has been suggested that persistent infection with LCM virus increases the incidence of lymphoid tumours (Traub, 1941; 1962). Sparck & Volkert (1965) have suggested the possibility of limiting the pathogenesis of infections involving immunological tolerance, by reversal of the tolerance by means of adoptive immunization.

Immunological tolerance to LCM virus has been shown to be incomplete, because small amounts of antibody are produced by LCM virus carrier mice (Section VII; Oldstone & Dixon, 1967; Benson & Hotchin, 1969). Circulating infectious virus-antibody complexes were found to be present in the carrier mice, and there is evidence that deposition of these complexes in the renal glomeruli results in chronic glomerulonephritis (Oldstone & Dixon, 1969). Infectious virus-antibody complexes are perhaps formed in adult mice infected with LCM virus, and the antibody would therefore permit maintenance of the infection, rather than aiding elimination of the virus and recovery. A similar situation might apply in a number of other viral infections and the development of immunological tolerance or paralysis may be unnecessary for the establishment of a persistent infection. The presence of infectious virus-antibody complexes has been demonstrated in lactic dehydrogenase
virus infection of mice (Notkins et al., 1966, 1968) and in Aleutian disease of mink (Porter et al., 1967, 1969). Other cases in which similar complexes might be involved include persistent infections with the viruses of rubella, equine infectious anaemia, hog cholera, African swine fever, mink encephalopathy, visna, scrapie and serum hepatitis. Investigations for the presence of infectious virus-antibody complexes in these conditions and studies of the nature of the antibody concerned in the formation of known complexes may provide further insights into the pathogenesis of "slow" virus diseases.

In primary footpad swelling in response to LCM virus infection of the footpad, the cellular and humoral immune responses probably both play a part, and obstruction of the lymphatic drainage of the foot increases the oedema. A hypersensitive footpad response to LCM virus has been described, in which footpad swelling occurs in sensitized mice after a challenge injection of LCM virus in the footpad. It can be divided into an early phase, which is probably due to the reaction between local viral antigen and circulating antibody, and a delayed phase, due to a cellular hypersensitivity reaction. The delayed response could be transferred to normal mice by the injection of sensitized spleen and lymph node cells, along with LCM virus, into the footpad. It provides a direct demonstration
of pathological changes due to the cellular immune response to LCM virus and enables the degree of cellular hypersensitivity to be determined. Further experiments utilizing this model of a local cellular hypersensitivity reaction should provide a clearer understanding of the pathogenesis of LCM virus infection. It may also be applicable to other anatomical situations, such as the brain after intracerebral injections. By analogy, transfer of lymphoid cells and antigen may provide an indication of the degree of cellular hypersensitivity to other microbial antigens, such as tuberculin, vaccinia virus and certain fungal and protozoal antigens.

Studies of the effects of Bordetella pertussis vaccine and Freund's complete adjuvant on the response of mice to infection with LCM virus showed that accelerated or delayed mortality may occur, depending on the route of infection. This is an indication that different factors or combinations of factors may be involved in the pathogenesis of the disease following different routes of infection. The mechanisms by which pertussis vaccine causes increased growth of LCM virus in the liver is one aspect which merits investigation. The full understanding of the results must await a greater knowledge of the causes of death in LCM virus infection. The different degrees of responsiveness of different strains of mice to infection
with LCM virus, even though the virus grew to the same extent in the tissues, emphasizes the importance of the intensity of the immune response in determining susceptibility to the infection.

The mouse-adapted, Armstrong strain of LCM virus behaves differently, on extraneural injection in mice, from the guinea pig-adapted WE3 strain virus. It was shown that mouse-adapted virus was not cleared as rapidly from the blood, probably due to less avid phagocytosis by cells of the reticuloendothelial system. In infant mice, extraneurally inoculated Armstrong strain virus did cause extensive infection of the organs, and this may be due to differences in the reticuloendothelial cells of infant and adult mice. In vitro, Armstrong strain virus infected peritoneal macrophages much less readily than WE3 strain virus. Presumably, some change occurs in the surface of the virus on growth in mouse cells, so that it is not so readily recognised as foreign by the reticuloendothelial cells. Studies of the nature of the differences between strains of LCM virus may provide an insight into the mechanisms of persistence of viral infections.
XIII. SUMMARY

The initiation and development of lymphocytic choriomeningitis (LCM) virus infection in the visceral organs and in the central nervous system of mice, after intravenous injection of the virus, have been studied by the immunofluorescence technique in conjunction with histological examination. Virus was rapidly taken up from the blood by cells of the reticuloendothelial system. In the liver, viral replication occurred in Kupffer cells before infection spread to hepatic cells and in the spleen, infection first occurred in perifollicular regions. Necrotic changes occurred in perifollicular regions in the spleen and in foci of infected cells in the liver. The necrotic lesions were shown to be fibrinoid in nature and the pathogenesis of the lesions has been investigated and discussed. They are thought to result from an interaction between sensitized lymphoid cells and infected cells, and antibody may contribute to their development. The thymus showed changes attributable to the action of adrenal corticosteroid hormones.

After virus had grown in the visceral organs, it spread via the blood stream to the central nervous system, where it infected the walls of blood vessels and passed...
through the meningeal vessels into the cerebrospinal fluid. Spread of infection through the choroid plexuses occurred later, but infection was not transmitted from cerebral blood vessels or from the cerebrospinal fluid into the brain substance. In infant mice, invasion of the central nervous system occurred much earlier and more readily than in adult mice and extensive infection developed in the brain substance as well as in the meninges.

The adoptive immunization of LCM virus carrier mice resulted in the production of antibodies. Virus free in the blood was neutralized, but the production of virus in the tissues remained unchanged and no pathological changes occurred. Possible explanations for the failure of transfer of a cellular immune response against LCM virus have been discussed. It has been found that immunological tolerance to LCM virus is not complete. Circulating infectious virus-antibody complexes were detected in LCM virus carrier mice and immunoglobulin deposits were present in the renal glomeruli.

Unsuccessful attempts have been made to develop a haemagglutination test for LCM virus.

The effects of Bordetella pertussis vaccine and Freund's complete adjuvant on the response of mice to infection with LCM virus have been studied. Delayed or accelerated mortality may occur, depending on the route
of infection. Possible mechanisms for these effects have been investigated and discussed.

The mechanisms involved in primary footpad swelling after footpad infection with LCM virus have been investigated. The immune response to the virus, as well as obstruction of the lymphatic drainage of the foot, appear to be involved. A hypersensitive footpad response to LCM virus has been described. An immediate phase of footpad swelling occurs, attributable to an antigen-antibody reaction, and this is followed by delayed swelling, due to the cellular immune response. The delayed reaction could be passively transferred to normal mice by means of lymphoid cells.

Studies have been made of the response of mice to infection with different strains of LCM virus. Mouse-adapted virus caused significant infection in adult mice only when injected intracerebrally, but in infant mice inoculation by any route resulted in extensive infection. Differences were demonstrated in the clearance of different strains of virus from the blood and in the ease of infection of macrophages in vitro.

The responses of different strains of mice to LCM virus infection have been compared. Virus grew to the same extent in the tissues, but the different strains of
mice showed different degrees of susceptibility to the infection, apparently depending on the intensity of the immune response.

The application of these findings to the present state of understanding of the pathogenesis of LCM virus infection has been discussed.
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