# THE PATHOGENESIS OF INFECTIOUS MYXOMATOSIS: THE MECHANISM OF INFECTION AND THE IMMUNOLOGICAL RESPONSE IN THE EUROPEAN RABBIT (*ORYCTOLAGUS CUNICULUS*).*

F. FENNER AND GWENDOLYN M. WOODROOFE.

*From the Department of Microbiology, John Curtin School of Medical Research, The Australian National University, Canberra, Australia.*

Received for publication April 14, 1953.

MYXOMATOSIS is a natural disease of *Sylvilagus brasiliensis* (syn. *minensis*) (Aragão, 1943), a native rabbit of Brazil, and probably of other rabbits of the same genus in other parts of America. In this species of rabbit it produces a localized tumour, not unlike the Shope fibroma in the cottontail or the European rabbit, and causes no appreciable mortality. In the European rabbit, on the other hand, it causes a generalized disease associated with an extremely high case-mortality rate, of the order of 99·5 per cent or more in previously unexposed populations (Fenner, Marshall and Woodroofe, 1953). Investigations on the mode of transmission of myxomatosis by mosquitoes showed that these insects, which were undoubtedly the principal vectors in the great Australian epizootics (Ratcliffe, Myers, Fennessy and Calaby, 1952), transmit the disease mechanically, and acquire virus only when they bite through epidermal cells containing a high concentration of virus (Fenner, Day and Woodroofe, 1952). Virus contained in the circulating blood appears to be unable to render the insects infectious. While the experiments upon which this conclusion was based were unequivocal, it did not appear easy to correlate the apparent frequency with which mosquitoes were infected with the comparative rarity in the field of rabbits with many large skin lesions, which would obviously provide a satisfactory source of virus for them. The experiments which form the basis of this report were undertaken to determine whether there was a more common source of virus in the skin than the single primary lesions and the rare multiple tumours, and to see if myxomatosis conformed in its mechanism of infection to other generalized virus diseases (Fenner, 1950). Concurrently, the immunological response of infected rabbits was studied, use being made of animals previously immunized with fibroma virus (Shope, 1932), and others infected with Hurst's neuromyxoma virus, a variant of reduced virulence (Hurst, 1937*b*).

## MATERIALS AND METHODS.

*Viruses.*

Two strains of myxoma virus were used, the classical South American strain (Fenner, Day and Woodroofe, 1952) and Hurst's "neuromyxoma" (Hurst, 1937*b*). Dr. E. Weston Hurst supplied the latter strain.

* Aided by a grant from the Rural Credits Development Fund of the Commonwealth Bank of Australia.

The OA strain of fibroma virus was provided by Dr. Richard E. Shope.

Stock virus was prepared from the subcutaneous tissue and testes of rabbits which had been given massive subcutaneous inoculations of the viruses. The gelatinous subcutaneous tissue was minced, ground thoroughly before and after freezing in a mortar, and taken up in normal rabbit serum. The suspension was distributed in a large number of ampoules, which were sealed and stored at −70°. This provided a source of virus of known high titre.

*Rabbits.*

Adult rabbits of various skin colours, weighing about 2·5 kg., were used. They had been bred in the animal house of the Walter and Eliza Hall Institute of Medical Research.

*Mosquitoes.*

Female *Aëdes aegypti* (*L*) mosquitoes were kindly provided by Dr. M. F. Day, of the Division of Entomology, Commonwealth Scientific and Industrial Research Organization. They were kept in a constant temperature room at about 27° and a relative humidity of 60–80 per cent.

*Titration of the virus-containing suspensions.*

Suspensions containing virus were titrated on the chorio-allantois of the developing chick embryo (Lush, 1937). Aliquots of the suspensions were stored in the dry ice cabinet, and if the chick embryo titrations were negative they were titrated by the intradermal inoculation of rabbits (Parker, 1940).

Chick embryos 11 or 12 days old were inoculated chorio-allantoically with 0·05 ml. of tenfold dilutions of the tissue suspensions, three eggs being used at each dilution. After three days' incubation at 35° the pocks on the membranes were counted, with the help of a binocular dissecting microscope if necessary. The titre is expressed as the average pock count per ml. of suspension.

Rabbits were inoculated intradermally in several sites with 0·1 ml. aliquots of the various dilutions used. Usually these were made in half log. or 2-fold (0·3 log.) steps. Readings were made on the third, fourth and fifth days, before the development of secondary lesions. The titre was calculated by the method of Reed and Muench (1938).

*Neutralizing antibody.*

Neutralizing antibody was titrated on the chorio-allantois of developing chick embryos. The technique of the test has been discussed in some detail in an earlier paper (Fenner, Marshall and Woodroofe, 1953). Undiluted serum, inactivated by heating for 30 min. at 56°, was mixed with the appropriate virus suspensions, and held at room temp. for 30 min. before inoculation of the chick embryos. All sera derived from any one experiment were titrated simultaneously.

*Complement fixation.*

The technique of the complement fixation test and the preparation of myxoma and control antigens was described earlier (Fenner, Marshall and Woodroofe, 1953). Serial 2-fold dilutions of each sample of serum were tested for complement-fixing activity in the presence of three 50 per cent haemolytic doses of complement and a standard dilution (usually 1/8 dilution of the 10 per cent suspension of infected chorio-allantoic membranes) of myxoma antigen. The indicator system of sensitized red cells was added after fixation had taken place overnight at 4°.

Soluble antigen in the serum was demonstrated by complement fixation tests. The sera were inactivated by heating at 62° for 15 min., and were diluted serially in 2-fold steps and titrated, in the presence of three 50 per cent haemolytic doses of complement, against a standard myxoma-immune serum, and normal rabbit serum, both at a dilution of 1/50. The indicator system was added after overnight fixation at 4°.

The anticomplementary activity of the serum samples was determined by the method described by Donnelley (1951). Undiluted sera were inactivated by heating at 62° for 15 min. To each was added an equal volume of excess complement (nine 50 per cent haemo-

lytic doses), and the system allowed to stand overnight at 4°. The " free " complement was then estimated by diluting out the mixture in 2-fold steps and adding to each dilution an equal volume of sensitized red cells. The end-point (50 per cent haemolysis) was read after incubation for 30 min. at 37°. Normal serum was used as a control and the anti-complementary activity of the sera was expressed as the percentage of complement fixed, taking the figure obtained with normal serum as zero.

*Comparison of the sensitivity of rabbits and eggs to the virus.*

Lush (1937) found that a suspension which produced an average count of 2·0 pocks on the chorio-allantoic membrane with an inoculum of 0·05 ml. of a $10^{-3}$ dilution killed 3/3 rabbits inoculated with 0·2 ml. of the $10^{-4}$ dilution and 1/3 of those inoculated with the $10^{-5}$ dilution ; i.e., the rabbit was somewhat more sensitive than the chick embryo. A more exact comparison was necessary for the present studies, and the following experiment was performed.

An ampoule of myxoma virus which had been stored at −70° and of which the approximate titre was known, was diluted in normal saline containing 10 per cent horse serum by 10-fold steps, using a clean 1·0 ml. pipette and 9 ml. blanks for each dilution. From $10^{-3}$, 2-fold dilutions were made to $10^{-4.5}$, and twenty twelve-day-old chick embryos were inoculated chorio-allantoically with 0·05 ml. of the dilutions $10^{-3.6}$ $10^{-3.9}$ $10^{-4.2}$ $10^{-4.5}$. From $10^{-5}$ 2-fold dilutions were made to $10^{-7.1}$, and rabbits were inoculated with 0·1 ml. in each of twenty sites, one rabbit being inoculated with each suspension from dilutions $10^{-5.0}$ to $10^{-7.1}$. The results of the pock count on the chorio-allantoics are shown in Table I, and the results

TABLE I.—*Pock Counts on Chorio-allantoic Membranes Inoculated with Dilutions of a Standard Suspension of Myxoma Virus.*

| Dilution. | Pock counts. | Mean. |
|---|---|---|
| $10^{-3.6}$ | 40, 49, 9, 36, 29, 25, 52, 34, 13, 8, 2, 2, 26, 34, 44, 31, 12 | 26·2 |
| $10^{-3.9}$ | 1, 10, 21, 2, 4, 8, 9, 4, 29, 1, 21, 12, 2, 9, 7, 14, 6, 12 | 9·6 |
| $10^{-4.2}$ | 1, 6, 0, 1, 12, 0, 1, 6, 11, 16, 14, 12, 5, 3, 1, 1, 4, 2, 5 | 5·3 |
| $10^{-4.5}$ | 1, 2, 1, 1, 2, 3, 11, 7, 0, 1, 2, 3, 2, 2, 3, 1, 7, 6, 3, 0 | 2·9 |

12-day-old embryo eggs incubated for 3 days at 35° after inoculation.
Inoculum volume = 0·05 ml.

TABLE II.—*Results of Intradermal Inoculation of Rabbits with Dilutions of a Standard Suspension of Myxoma Virus.*

| Dilution. | Positive. | Negative. | Percentage positive. |
|---|---|---|---|
| $10^{-5.0}$ | 20 | 0 | 100 |
| $10^{-5.3}$ | 19 | 1 | 95 |
| $10^{-5.6}$ | 19 | 1 | 95 |
| $10^{-5.9}$ | 15 | 5 | 75 |
| $10^{-6.2}$ | 14 | 6 | 70 |
| $10^{-6.5}$ | 4 | 16 | 20 |
| $10^{-6.8}$ | 1 | 19 | 5 |
| $10^{-7.1}$ | 1 | 19 | 5 |

Inoculum = 0·1 ml. of dilutions used for egg titration (Table I).

of the rabbit inoculation in Table II. The latter were very similar to those reported by Parker (1940). If all the results in the egg experiment were pooled the figure was obtained of $10^{-6.25}$ pock-producing particles per ml. in the suspension, compared with a Reed and Muench titre of $10^{-7.27}$ $I.D._{50}$ per ml. in the rabbit. Since the mean number of infectious particles in one $I.D._{50}$ is 0·693, the rabbit titre is equivalent to $10^{-7.10}$ infective particles per ml. For the purposes of present experiments a comparative figure only was required, and in all the subsequent experiments the egg titre (infective particles per ml.) has been converted to $I.D._{50}$ per ml. (rabbit) by multiplying the former by 10.

## RESULTS.

### *Distribution of Virus in Rabbits following Infection.*

The plan of the first experiment followed rather closely that previously employed in studies on mousepox (Fenner, 1948*a*).

Twenty adult male rabbits were inoculated intradermally in a marked area in the left flank, in the centre of an area about 3 cm. in diameter from which the hair had been removed by plucking. Simultaneously the virulent myxoma virus used for the inoculations was titrated in eggs and in a rabbit. The dose of 0·1 ml. of the $10^{-4\cdot7}$ dilution was found to contain approximately 20 $I.D._{50}$ of virus.

At daily intervals one or two rabbits were bled from the heart, some of the blood being heparinized and the remainder used for serum collection. They were then exsanguinated by washing out the vascular system with 4 l. sterile normal saline through a cannula which was inserted through the left ventricle and into the aorta.

The virus content of the heparinized blood was determined with whole blood; washed cells and plasma were also tested separately. "Plasma" consisted of the final supernate after three cycles of centrifugation, each for 30 min. in an angle centrifuge; "washed cells" comprised the sediment of the first centrifugation washed once with normal saline, deposited by centrifugation for 30 min. and then made up to original volume of the blood in normal saline.

The skin was removed from the inoculated site, and from an equivalent area on the other side of the body. The hair was plucked from the latter site after the rabbit had been exsanguinated. The crural lymph-nodes beneath the skin at these two sites were removed, then the left testis, the spleen and the left lung. All organs and pieces of tissue were weighed and ground with sand in mortars which had been cooled at −20°. After the preliminary grinding the tissues were frozen at −20° before re-grinding. The final suspension was taken up in a volume of normal horse serum saline equivalent to the weight of the sample, or ten times this weight, depending on the size of the sample; a portion was ampouled and stored at −70°, and the rest was titrated in either or both eggs and the rabbit. The results are summarized in Table III. Certain data have been extracted from this table and used to construct Fig. 1. Each rabbit was carefully examined for abnormal physical signs, and from the 5th day onwards washings were made of the conjunctival fluid.

It is apparent that in myxomatosis, as in mousepox and other generalized viral and bacterial diseases, there is a stepwise invasion of the organs. After an initial period of multiplication in the site of inoculation, virus reached and probably multiplied in the draining lymph node, in which a small amount of virus could be demonstrated 48 hr. after inoculation. By the next day small amounts of virus could be detected in a number of organs and in the cells of the circulating blood. There was a steady rise in titre in all affected organs. Virus was first demonstrated in the testis and "normal" skin a day later than in the blood and central organs. The titre in the whole blood and washed cells was identical throughout, and virus was never demonstrated in the plasma. In the day or so before death the amount of detectable virus in certain organs (blood, spleen, etc.) fell somewhat.

Daily examination of the animals showed that changes were present in both the inoculated skin site (redness, thickening) and in the regional lymph node

TABLE III.—*Titre of Myxoma Virus in Various Organs and Tissues at Intervals after Inoculation, Expressed as the $Log_{10}$ of the I.D.$_{50}$ per ml. of Fluid or g. of Tissue.*

| | Days after inoculation. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1. | 2. | 3. | 4. | 5. | 6. | 7. | 8. | 9. | 10. |
| Skin-inoculation site | 2·6, – | 2·8, 4·7 | 6·5, 5·9 | 7·2, 7·3 | 6·7, 7·3 | 7·6 | 8·3 | 8·3, 8·4 | 8·4, 8·0 | 7·7 |
| " Normal " skin | — | — | 0, 0 | 0, 2·4 | 2·1, 3·9 | 5·5 | 4·7 | 6·4, 7·2 | 7·1, 5·7 | 7·1 |
| Lymph node : inoculated side | 0, 0 | 2·6, 2·6 | 7·7, 5·8 | 7·3, 7·4 | 6·1, 6·8 | 7·9 | 6·4 | 6·9, 7·8 | 6·9, 6·7 | 6·8 |
| Lymph node : " normal " side | — | 0, 0 | 0, 2·3 | 3·1, 4·5 | 3·2, 3·1 | 4·5 | 4·8 | 4·5, 6·9 | 6·1, 5·1 | 6·6 |
| Whole blood | 0, 0 | 0, 0 | 2·9, 2·8 | 3·9, 3·8 | 1·6, 3·8 | 3·3 | 2·3 | 3·4, 5·5 | 2·0, 1·8 | — |
| Washed blood cells | 0, 0 | 1·3, 0 | 2·8, 2·7 | 3·3, 3·6 | 1·8, 3·6 | 3·3 | 1·9 | 3·0, 5·6 | 2·3, 1·8 | — |
| Plasma | 0, 0 | 0, 0 | 0, 0 | 0, 0 | 0, 0 | 0 | 0 | 0, 0 | 0, 0 | — |
| Lung | 0, 0 | 0, 0 | 3·2, 2·5 | 4·4, 4·3 | 4·3, 4·1 | 6·0 | 5·6 | 5·1, 7·4 | 4·3, 3·6 | 7·0 |
| Spleen | 0, 0 | 0, 0 | 3·1, 2·7 | 4·5, 4·2 | 4·2, 3·9 | 4·2 | 3·2 | 0, 5·5 | 0, 2·9 | 7·0 |
| Testis | 0, 0 | 0, 0 | 0, 0 | 0, 2·4 | 3·9, 4·5 | 5·1 | 7·4 | 8·1, 8·0 | 8·3, 7·4 | 7·4 |
| Conjunctival washings | — | — | — | — | 4·1, 4·2 | 4·7 | 5·5 | 5·1, 6·3 | 5·9, 6·6 | 7·9 |

— = not determined. 0 = < 10 I.D.$_{50}$ (lowest amount detectable).

TABLE IV.—*Development of Neutralizing and Complement-fixing Antibodies, and of Complement-fixing Antigen, in Rabbits Injected in 20 Sites with Small Doses of Myxoma Virus (20 I.D.$_{50}$) and Neuromyxoma Virus (10 I.D.$_{50}$).*

| | Neutralizing antibody.[1] | | | | | | Complement-fixing antibody.[2] | | | | | | Complement-fixing antigen.[3] | | | Anticomplementary activity.[4] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Neuro-myxoma. | | Myxoma. | | | Fibroma then myxoma. | Neuro-myxoma. | | Myxoma. | | | Fibroma then myxoma. | Myxoma. | | | Neuro-myxoma. | | Myxoma. | | | Fibroma then myxoma |
| Days after inoculation. | 255. | 257. | 259. | 260. | 261. | 262. | 255. | 257. | 259. | 260. | 261. | 262. | 259. | 260. | 261. | 255. | 257. | 259. | 260. | 261. | 262. |
| 1 day | 0 | 0 | 0 | 0 | 0 | 67 | 0 | 0 | 0 | 0 | 0 | 40 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 days | 0 | 0 | 0 | 0 | 0 | 65 | 0 | 0 | 0 | 0 | 0 | 40 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 „ | 40 | 42 | 0 | 5 | 45 | 76 | 0 | 0 | 0 | 0 | 0 | 40 | 0 | 0 | 0 | 0 | 0 | 0 | 13 | 33 | 38 |
| 6 „ | 35 | 38 | 32 | 0 | 0 | 96·2 | 0 | 0 | 0 | 0 | 0 | 640 | 0 | 0 | 0 | 7 | 0 | 0 | 88 | 78 | 33 |
| 7 „ | 0 | 0 | 0 | 0 | 0 | 98·2 | 0 | 0 | 0 | 0 | 0 | 640 | 2048 | 0 | 0 | 43 | 43 | 69 | 88 | 86 | 96 |
| 8 „ | 0 | 0 | 0 | 0 | 0 | 98·3 | 0 | 0 | 0 | 0 | 0 | 640 | 2048 | 2048 | 2048 | 50 | 76 | 75 | 94 | 94 | 98 |
| 9 „ | 29 | 29 | 29 | 50 | 0 | 98·7 | 0 | 0 | 0 | 0 | 0 | 1,280 | 2048 | 4096 | 4096 | 50 | 86 | 92 | 94 | 95 | 92 |
| 10 „ | 72 | 65 | † | † | † | 99·3 | 0 | 0 | † | † | † | 1,280 | † | † | † | 79 | 90 | † | † | † | 92 |
| 11 „ | 85 | 84 | .. | .. | .. | 98 | 160 | 80 | .. | .. | .. | 1,280 | .. | .. | .. | 50 | 77 | .. | .. | .. | 92 |
| 12 „ | 90 | 87 | .. | .. | .. | 98 | 160 | 320 | .. | .. | .. | 640 | .. | .. | .. | 47 | 72 | .. | .. | .. | 88 |
| 14 „ | 96·5 | 95·6 | .. | .. | .. | 98·3 | 640 | 640 | .. | .. | .. | 2,560 | .. | .. | .. | 14 | 72 | .. | .. | .. | 54 |
| 15 „ | 96·7 | 96 | .. | .. | .. | 99 | 640 | 640 | .. | .. | .. | 2,560 | .. | .. | .. | 14 | 72 | .. | .. | .. | 50 |
| 21 „ | 97·2 | 97 | .. | .. | .. | 98·7 | 2560 | 2560 | .. | .. | .. | 10,240 | .. | .. | .. | 7 | 72 | .. | .. | .. | 34 |
| 28 „ | 98·7 | 96·7 | .. | .. | .. | 98·3 | 5120 | 5120 | .. | .. | .. | 10,240 | .. | .. | .. | 0 | 50 | .. | .. | .. | 36 |

[1] Neutralizing antibody : percentage reduction in pock count on chorio-allantoic membrane.
[2] Complement-fixing antibody : reciprocal of titre.
[3] Complement-fixing antigen : reciprocal of titre. Neuromyxoma and fibroma-myxoma rabbits : no complement-fixing antigen at any stage.
[4] Anticomplementary activity $= 100 - \frac{100 \times \text{titre in presence of anticomplementary substance (serum sample)}}{\text{complement titre (normal serum)}}$.

† = Death.

(enlargement, engorgement) on the 3rd day after inoculation. Slight thickening of the eyelids was detectable on the 5th day, and virus could then be isolated from washings of conjunctival fluid. On the 6th day there were visible on the skin opposite the inoculation site a number of macules about 3 mm. diameter, and examination of the rest of the carcass showed that these occurred generally.

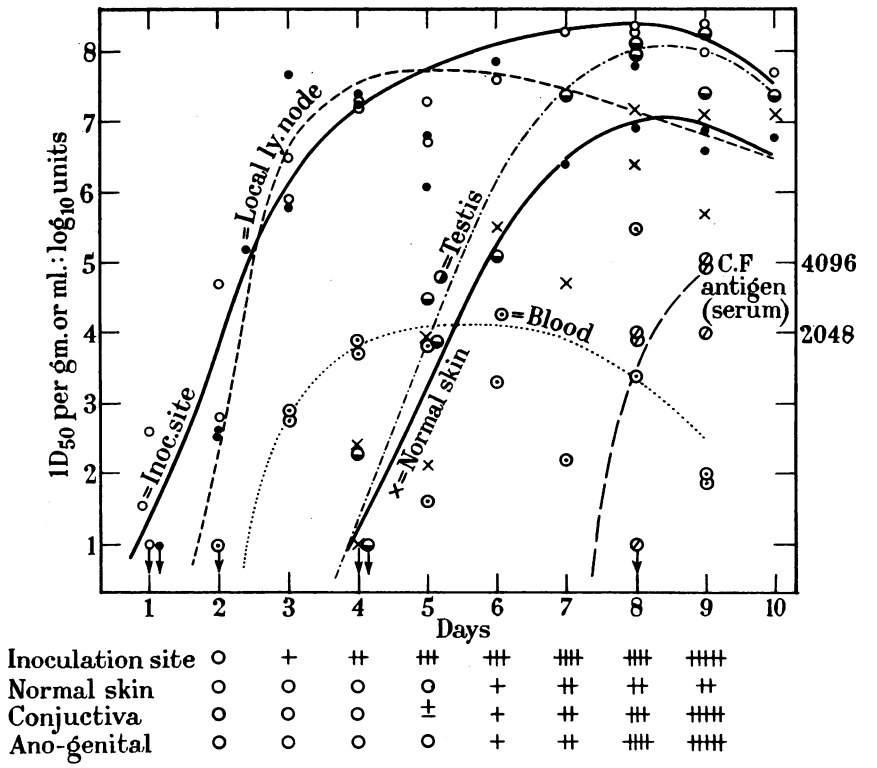



FIG. 1.—Concentrations of myxoma virus and of soluble complement-fixing antigen (ordinates on right = reciprocal of titre) in various tissues and organs of rabbits at intervals after the intradermal inoculation of 20 $I.D._{50}$ of virus, correlated with the development of abnormal physical signs.

± to +++++ = arbitrary grading of severity of physical signs.
"Normal skin" = skin on a portion of the body distant from the inoculation site.
Arrowed points = < 10 $I.D._{50}$/gm. (lowest amount detectable).

They became larger and more thickened with the passage of time. There is no doubt that they constitute a true secondary rash closely analogous to that characteristic of the acute exanthemata. Fig. 2 illustrates this rash in a rabbit 8 days after inoculation with 20 $I.D._{50}$ of virus. Areas of engorgement were noticed in the body of the testis from the 6th day onwards. The severity of all these signs increased until the time of death.

*The Immunological Response in Infected Rabbits.*

The serum collected from the 20 animals used for the experiment was tested for complement-fixing and neutralizing antibodies, complement-fixing antigen,

and anticomplementary activity. All sera were tested together, so that results would be comparable. It appeared that the animals just began to produce antibodies a day or so before they died. Although no free complement-fixing antibody appeared, the occurrence of anticomplementary activity in the serum from the 7th day onwards was evidence of the presence of antigen-antibody union *in vivo*.

The immunological response was followed with greater ease in the next experiment, in which both myxoma and neuromyxoma viruses were used. This experiment was planned differently from that just described, the object being to trace the immunological response more clearly, and to compare the clinical symptoms and rate of growth of neuromyxoma virus with the fully virulent myxoma virus. The latter features will be discussed in a later paper.

With each strain of virus 3 rabbits were inoculated intradermally on marked sites in the carefully plucked skin of each flank, with a total of 10 inoculations each of 0·1 ml., the inoculum containing about 20 $I.D._{50}$ of myxoma virus and 10 $I.D._{50}$ of the neuromyxoma virus. The titres of the inocula were determined by making 10 inoculations of each of 4 appropriately selected 2-fold dilutions, and calculating the titre by the method of Reed and Muench (1938). One of the animals inoculated with myxoma virus had been inoculated with the OA strain of fibroma virus 8 months earlier. Each animal was bled daily.

All the normal rabbits infected with myxoma virus died, but the survival of those infected with neuromyxoma, and the animal (R262) which had been previously inoculated with fibroma virus, allowed a more satisfactory study to be made of the immunological response to infection. The development of neutralizing and complement-fixing antibodies and the complement-fixing antigen content of the daily samples of serum are shown in Table IV.

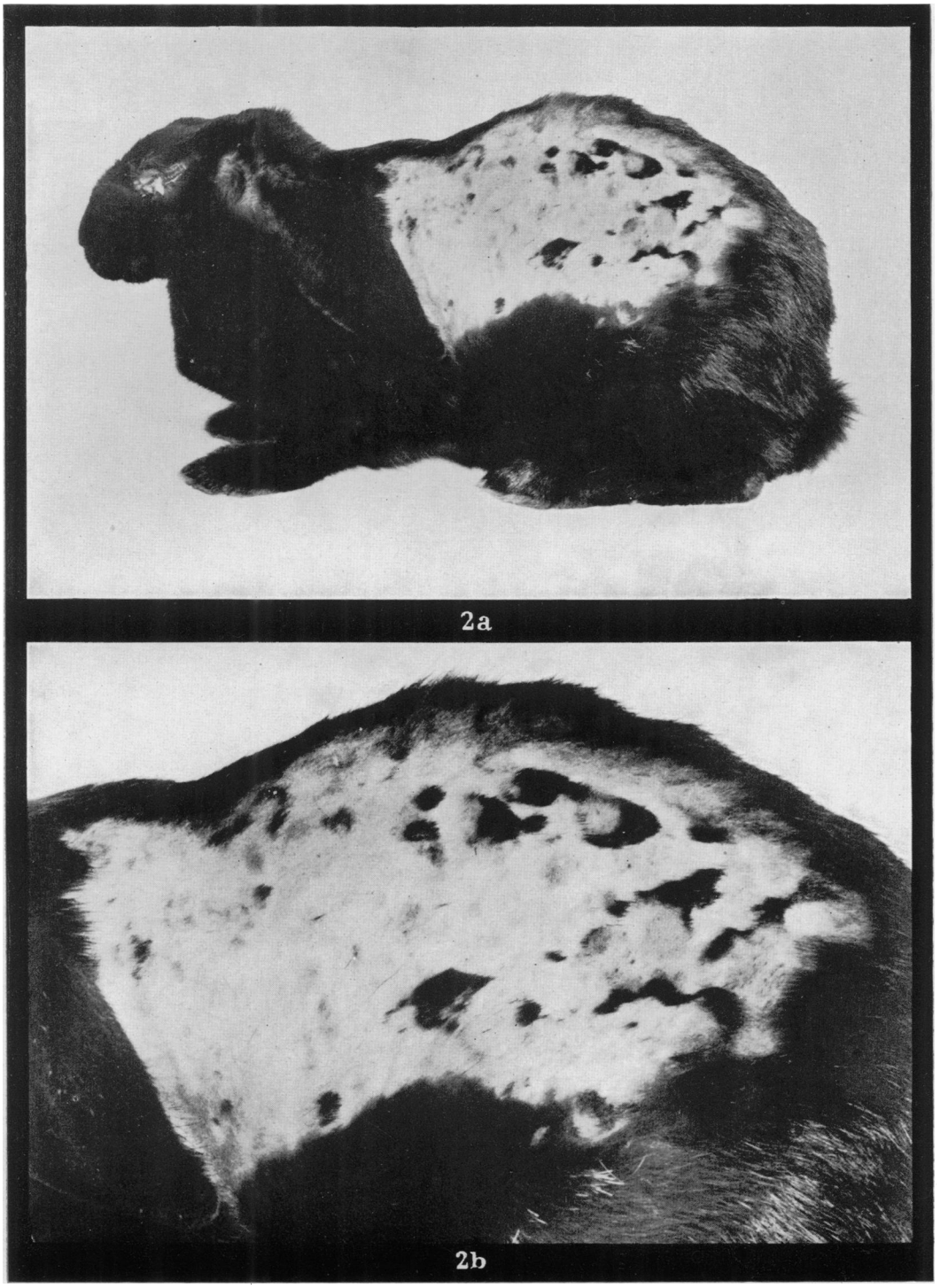
As in the previous experiment, the normal rabbits infected with the fully virulent myxoma virus (R259, R260, R261) produced no detectable free antibody before death, but on the 7th and 8th days complement-fixing antigen appeared in the serum in large amounts. From the 6th and 7th days there was a considerable degree of anticomplementary activity in the serum, which can be regarded as an index of the presence in the serum of an antigen-antibody complex (Donnelley, 1951).

In the rabbits infected with neuromyxoma the development of antibody could be clearly followed. Complement-fixing antigen could not be detected at any stage, but anticomplementary activity appeared on the 7th day and persisted until the 28th day. A significant rise in neutralizing antibody occurred

EXPLANATION OF PLATE.

FIG. 2A.—Photograph of a rabbit which had been inoculated intradermally in the right flank 8 days earlier with 20 $I.D._{50}$ of myxoma virus. The area on the left flank was plucked just before the photograph was taken, and shows, besides some patches of black hair, numerous secondary papules which were red in colour and varied from 2–10 mm. in diameter. These are seen more clearly in the enlargement of the plucked area of skin (Fig. 2B). The generalized swelling of the face, closure of the eyes, with a purulent conjunctival discharge, and thickening and drooping of the ears are characteristic of advanced myxomatosis. This rabbit was used immediately afterwards for feeding mosquitoes on various sites (Table VII). Photograph by D. Wilson.

FIG. 2B.—Enlargement of plucked area of skin on rabbit shown in Fig. 2A. The jet black areas are patches of black hair; the scattered grey areas of different sizes are the papules of the generalized rash.



2a

2b

on the 10th day and continued until the 14th day, after which there was only a slight rise. Complement-fixing antibody appeared in both rabbits on the 11th day and continued to rise until a very high titre was attained by the 28th day.

The fibroma-vaccinated rabbit showed an accelerated antibody response. Complement-fixing antigen never appeared in the serum, but anticomplementary activity appeared suddenly on the 5th day and was present thereafter, falling slightly at the 28th day. There was a residual level of about 70 per cent reduction in pock count on the egg membrane and 40 in complement-fixing antibody, due to the fibroma inoculation 8 months earlier. A sudden rise in antibody titre, tested by both techniques, occurred on the 6th day, and thereafter the titres rose to very high levels. The highest titre of complement-fixing antibody was not reached until the end of the 3rd week.

*The distribution of virus in the blood.*

The experiments just described confirmed previous reports (Hobbs, 1928; Rivers and Ward, 1937) that although soluble antigen may appear to a high titre in the serum, virus particles are associated with the particulate components of the blood and do not occur free in the plasma. An attempt was made to determine what cellular elements contained the bulk of the circulating virus. A rabbit which had been inoculated subcutaneously in several sites with large doses of myxoma virus was exsanguinated on the 5th day, half the blood being mixed with heparin and half defibrinated by stirring it with roughened glass rods. Various fractionation procedures were then carried out and the final products examined for virus by titration in eggs or rabbits, and for cellular composition by total counts of the erythrocytes and leucocytes, and the examination of smears stained with Leishman's stain.

The particulate elements were separated from the plasma by centrifugation at 2° for $\frac{1}{2}$ hr. at 2000*g* and the operation was repeated once with the plasma, the final supernatant then being tested for its virus content. The cellular sediment was washed once with saline, deposited in the centrifuge, and re-suspended to the original blood volume before determination of its cellular and virus content. One sample of heparinized blood was then repeatedly centrifuged, the leucocyte-rich buffy layer being separated from the erythrocyte sediment. Owing to a technical error the final product of the concentrated buffy coat was discarded, and tests could therefore be made only of the lowest layer from which the majority of the leucocytes, and the platelets, had been separated. Another sample of heparinized blood was mixed with fibrinogen and the sedimentation of the erythrocytes accelerated, as described by Minor and Burnett (1948). This procedure was repeated once, and the supernatant fluid, containing most of the leucocytes and platelets and few erythrocytes, was examined. Results are shown in Table V.

In order to appreciate the association of virus titres and the concentrations of different particulate components of the blood, the results have been expressed as the percentage of cells of the various types present in the different samples compared with the original specimen of heparinized blood and the percentage of virus. As in previous experiments, free virus could not be demonstrated in the plasma. Procedures which eliminated platelets or erythrocytes had a negligible effect (considering the relative inaccuracy of the virus titrations) on the virus

TABLE V.—*Cellular Composition and Virus Content of Specimens of Blood Subjected to Various Treatments.*

| Preparation. | Erythrocyte count (per c.mm.) | Leucocyte count (per c.mm.). | Differential leucocyte count (per cent). Granulocytes. | Lymphocytes. | Monocytes. | Platelets. | Virus titre ($log_{10}$ I.D.$_{50}$ per ml.). |
|---|---|---|---|---|---|---|---|
| Heparinized whole blood | $6{\cdot}7\times10^6$ | $10\times10^3$ | 31 | 62 | 7 | +++ | 4·5 |
| Defibrinated blood | $6{\cdot}4\times10^6$ | $2{\cdot}4\times10^3$ | 4 | 89 | 7 | 0 | 4·3 |
| Plasma | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Washed cells | $5{\cdot}4\times10^6$ | $5{\cdot}9\times10^3$ | 35 | 57 | 8 | +++ | 4·3 |
| Sediment after repeated centrifugation | $3{\cdot}1\times10^6$ | $3\times10^3$ | .. | 100* | .. | 0 | <1·0 |
| Supernatant after accelerated sedimentation | $2{\cdot}9\times10^6$ | $4{\cdot}2\times10^3$ | 12 | 74 | 14 | +++ | 4·6 |

* Insufficient leucocytes present in smear to enable count to be made—all those seen were lymphocytes.

TABLE VI.—*Percentages of Erythrocytes, Leucocytes and Platelets, and of Virus, in Specimens of Blood Subjected to Various Treatments.*

| Preparation. | Erythrocytes. | Granulocytes. | Lymphocytes and monocytes. | Platelets. | Virus. |
|---|---|---|---|---|---|
| Heparinized blood | 100 | 100 | 100 | 100 | 100 |
| Defibrinated blood | 95 | 3 | 34 | 0 | 95 |
| Sediment after repeated centrifugation | 46 | 0 | 4 | 0 | <2 |
| Supernatant after accelerated sedimentation | 0·4 | 17 | 46 | 100 | 100 |

concentration, whereas removal of the leucocytes caused a great fall in the virus titre. It appeared that the virus was probably associated with lymphocytes rather than granulocytes, for a considerable fall in the percentage of granulocytes, caused by defibrination, had a negligible effect on the virus titre.

*Infectivity for Mosquitoes of the Secondary Skin Lesions in Advanced Cases of Myxomatosis.*

The importance of the secondary skin lesions and the lesions of the face and ears, as sources of virus which might contaminate the mouthparts of mosquitoes and render them infective, was investigated by allowing *Aëdes aegypti* to bite in these sites, and subsequently determining their infectivity for other rabbits. A rabbit was inoculated intradermally in the left flank with 20 I.D.$_{50}$ of myxoma virus. Eight days later, when the disease was far advanced (see Fig. 2), female mosquitoes which had been starved for 24 hr. were given the opportunity to bite at various sites. Each mosquito was kept in a small numbered vial. About 20 mosquitoes bit through each of the following sites: the primary lesion, at the inoculation site, the thickened skin of the upper eyelid, secondary lesions on the body, and the thickened base of the ears. Each mosquito was given an oppor-

tunity to bite a marked site on the back of a normal rabbit on the day after the " acquisition " feed : 68 of the 73 mosquitoes fed on this occasion. The results are shown in Table VII.

TABLE VII.—*Infectivity of Mosquitoes which had Bitten through Skin Lesions in Various Parts of a Rabbit with Advanced Myxomatosis.*

| Site of acquisition feed. | Results. Proportion positive. | Percentage positive. |
|---|---|---|
| Primary lesion . . . . | 11/11 | 100 per cent |
| Swollen eyelids . . . . | 14/18 | 78 „ |
| Swollen base of ears . . . | 11/19 | 58 „ |
| Secondary skin lesions on flank . | 3/20 | 15 „ |

Tests were made 1 day after the " acquisition " feed.

As was expected, all mosquitoes which probed through the large primary lesion at the inoculation site became infective, and there is reason to believe that the advanced secondary tumours which sometimes occur would also be rich sources of virus. More important, from the point of view of the epidemiology of myxomatosis, is the high proportion of positive results recorded when mosquitoes bit the eyelids or base of the ears of a rabbit in an advanced stage of the disease. Under natural conditions it is probable that these lesions, which are invariably present in rabbits suffering from advanced myxomatosis, are the most important source of virus for mosquitoes. The smaller papules of the secondary rash yielded a smaller proportion of infective bites ; under natural conditions they would also be less accessible to mosquitoes than the facial lesions.

## DISCUSSION.

The results reported in this paper illustrate the general validity of the hypothesis outlined earlier concerning the mechanism of infection in generalized infectious diseases (Fenner, 1950). Myxomatosis in the European rabbit closely resembles mousepox in the laboratory mouse, in the occurrence of a stepwise infection of the primary lesion, the regional lymph node, generalization through the blood stream and later localization in certain areas (skin, testis) with the development of a " secondary rash". It differs from mousepox in the simultaneous appearance of virus in the circulating blood and a number of internal organs. Hurst's (1937*a*) histological studies provide an explanation of this. He reported the existence of distinctive lesions of the small arterioles in all organs of the body. The vascular tree may thus function as the " internal focus " of multiplication, analogous to the spleen and liver in mousepox, and after multiplication there deposition may occur in epithelial cells of the skin, in the testis, and elsewhere. The time intervals between the demonstration of virus at the site of inoculation, in the regional lymph node and in the viscera depend upon the presence of infective virus in the tissues examined. If there is an early non-infective stage in the growth of myxoma virus, as there is in influenza virus (Hoyle, 1948 ; Henle, 1949), and current experiments with myxoma virus on the chorio-allantois suggest that there is, the absolute interval between deposition of virus in the skin and its presence in the local lymph node would be shorter

than our results suggest. Further, an interval such as we have found can be demonstrated only if very small infective doses are used, so that local multiplication must occur before sufficient virus is available to be detected in the lymph node, etc. If large infective doses are used virus will be found in the local lymph node within a few minutes of the inoculation (McMaster and Hudack, 1935), but our aim has been to imitate as closely as possible the course of events which occurs in the naturally transmitted disease.

It is apparent from the final experiment that the facial lesions, an invariable development in the advanced stages of myxomatosis and a site easily accessible to mosquitoes, probably provide the principal source of virus available for the contamination of the mouth parts of insect vectors under field conditions. The primary lesion, and the secondary skin lesions on the body if they were well developed and relatively free of hair, would constitute another important source of virus.

These experiments provide additional evidence of the unsuitable nature of the blood as a source of virus for mosquitoes, if transmission is, as we believe, mechanical in nature. The plasma is free of virus, all the circulating virus being associated with particulate elements in the blood; principally, it appears, with the lymphocytes. The situation is similar to that described in vaccinia (Smith, 1929), rinderpest (Daubney, 1928) and fowl plague (Todd, 1928). Although the plasma is usually free of virus elementary bodies, it contains a high concentration of soluble antigen in the later stages of infection with virulent myxoma virus.

The serological response in infections with myxoma virus closely parallels that occurring in other generalized virus diseases. The first indication of the presence of antibody in the serum was usually the appearance of anticomplementary activity. Donnelley (1951) discussed the significance of the anticomplementary activity of extracts of mouse lungs in the early stages of experimental influenza. She found that the rise of anticomplementary activity preceded the appearance of free antibody in the serum, and when free antibody appeared the degree of anticomplementary activity declined. The same was true of neuromyxoma when daily tests were made on the serum. With virulent myxoma virus the animals never lived long enough to permit the demonstration of free antibody in the serum, but there was a sharp rise in anticomplementary activity about the 6th or 7th day. In the rabbits infected with neuromyxoma virus neutralizing antibody appeared 1 day before complement-fixing antibody, and both rose rather slowly to reach a high titre. As free antibody was not detected until the 11th day, whereas the rabbits with myxomatosis had all died on the 9th day, it is not surprising that free antibody was not detected in the latter animals. The persistence of these antibodies in rabbits which had recovered from myxomatosis has been described earlier (Fenner, Marshall and Woodroofe, 1953).

## SUMMARY.

Myxomatosis in the European rabbit is a generalized disease, in which the virus spreads in a stepwise fashion from the site of inoculation, through the local lymph node to the circulation. After a period of multiplication in an internal focus, which may be the walls of the small arterioles, secondary dissemination occurs in the skin and elsewhere. Multiplication of this secondarily distributed virus causes the development of generalized lesions of the skin, consisting of

scattered small papules over the body and generalized thickening of the face and the loose tissues in the anogenital region.

The primary and secondary skin lesions, especially the thickened tissues around the eyes and the base of the ears, as well as the papules on the body, were shown to be rich sources of virus, in terms of the ability of mosquitoes to acquire infection by biting through such lesions.

The virus particles which circulate during the viraemic stage of the disease are associated with the leucocytes, probably with the lymphocytes, and in the late stages of the disease soluble antigen appears at high titre in the serum.

The serological response was followed in rabbits infected with neuromyxoma virus. Soluble antigen did not appear in the serum in infections with this strain, but the serum became anticomplementary on about the seventh day, and on the 11th day free antibody appeared and rose steadily to reach a high level by the 14th day.

We are grateful to Sir Macfarlane Burnet, Director of the Walter and Eliza Hall Institute for Medical Research, for making laboratory space and facilities available to us pending the completion of the University laboratories in Canberra.

Miss Grace Hubbard gave valuable technical assistance, and Mr. D. Wilson, Division of Entomology, Commonwealth Scientific and Industrial Research Organization, took the photographs.

## REFERENCES.

ARAGÃO, H. DE B.—(1943) *Mem. Inst. Oswaldo Cruz*, **38**, 93.
DAUBNEY, R.—(1928) *J. comp. Path. Therap.*, **41**, 228.
DONNELLEY, M.—(1951) *Aust. J. exp. Biol.*, **29**, 137.
FENNER, F.—(1948) *J. Path. Bact.*, **60**, 529.—(1950) *Med. J. Aust.*, **2**, 813.
*Idem*, DAY, M. F., AND WOODROOFE, G. M.—(1952) *Aust. J. exp. Biol.*, **30**, 139.
*Idem*, MARSHALL, I. D., AND WOODROOFE, G. M.—(1953) *J. Hyg., Camb.*, in press.
HENLE, W.—(1949) *J. exp. Med.*, **90**, 1.
HOBBS, J. R.—(1928) *Amer. J. Hyg.*, **8**, 800.
HOYLE, L.—(1948) *Brit. J. exp. Path.*, **29**, 390.
HURST, E. W.—(1937*a*) *Ibid.*, **18**, 1.—(1937*b*) *Ibid.*, **18**, 15.
LUSH, D.—(1937) *Aust. J. exp. Biol.*, **15**, 131.
MCMASTER, P. D., AND HUDACK, S. S.—(1935) *J. exp. Med.*, **61**, 783.
MINOR, A. H., AND BURNETT, L.—(1948) *Blood*, **3**, 799.
PARKER, R. F.—(1940) *J. exp. Med.*, **71**, 439.
RATCLIFFE, F. N., MYERS, K., FENNESSY, B. V., AND CALABY, J. H.—(1952) *Nature, Lond.*, **170**, 7.
REED, L. J., AND MUENCH, H.—(1938) *Amer. J. Hyg.*, **27**, 493.
RIVERS, T. M., AND WARD, S. M.—(1937) *J. exp. Med.*, **66**, 1.
SHOPE, R. E.—(1932) *Ibid.*, **56**, 803.
SMITH, W.—(1929) *Brit. J. exp. Path.*, **10**, 93.
TODD, C.—(1928) *Ibid.*, **9**, 19.