Br. J. exp. Path. (1971) **52**, 408.

DELAYED ONSET OF ENCEPHALITIS IN MICE PASSIVELY IMMUNISED AGAINST SEMLIKI FOREST VIRUS

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Received for publication January 29, 1971

SUMMARY.—After a delay of 2 to 10 weeks or longer, clinical encephalitis developed in 15 per cent of mice which were protected against the acute stage of Semliki Forest virus (SFV) infection by antiserum. Some of these mice had high titres of SFV in their brains together with lesions of acute encephalitis while the others had subacute lesions and no virus was detected. When brains of apparently healthy mice which had survived the acute stage of SFV infection were examined, 90 per cent appeared to be histologically normal, but residual lesions of encephalitis were found in most of the remainder. Although no virus was detected in the brains of apparently healthy mice which survived the acute stage, the development of delayed encephalitis appeared to be due to persistence of SFV from the original infection. Examination of the sera of mice treated with antiserum suggested that most failed to develop active immunity.

RECOVERY from certain virus infections such as herpes and measles may not be so complete as it appears and may in fact be complicated by the persistence of virus. When virus persists, the possibility of a resurgence of infection remains a potential hazard: this would seem to be true whether recovery has been effected naturally or with the aid of serum or chemotherapy. In recovery from viral encephalitis the immune response is directly related to the inflammatory reaction and is usually beneficial (Zlotnik, Smith, Grant and Peacock, 1970). However evidence has been produced to show that the effects of the immune response may be equivocal or indeed adverse. Thus Webb, Wight, Platt and Smith (1968) reported that treatment of Langat virus infection of mice with antiserum could increase the incidence of clinical encephalitis although the time of onset was somewhat delayed, and Berge, Gleiser, Gochenour, Miesse and Tigertt (1961) showed that administration of antiserum to mice with Venezuelan equine encephalomyelitis accelerated the appearance of cerebral inflammatory lesions but prolonged the incubation period and the length of survival. Similarly Boulter (unpublished observations) found that although rabbits given SFV by the respiratory route could be protected by antiserum there were some late deaths due to encephalitis. SFV normally produces an acute fatal encephalomyelitis in mice (Seamer, Randles and Fitzgeorge, 1967), but the present report describes an encephalitis which developed in some mice several weeks after they had apparently been successfully protected against SFV infection with antiserum.

MATERIALS AND METHODS

Virus.—SFV from the American Type Culture Collection (VR67) in the 13th mouse brain passage since the original isolation by Smithburn and Haddow (1944) was used. The virus content of mouse brains was assayed on chick embryo monolayer cultures as previously

described (Seamer *et al.*, 1967); 1 plaque forming unit (pfu) was found to be equivalent to 2·4 mouse i.c. LD_{50}.

Antisera.—Four batches of antisera against SFV were prepared in rabbits. The neutralising titres of these antisera were 5·4, 5·2, 5·0 and 4·6 respectively, and no differences were observed between sera in the various experiments. Neutralising titres of mouse sera were assayed by mixing 2-fold dilutions of the inactivated sera with equal volumes of a suspension containing 75–100 pfu SFV. Duplicate sets of each mixture were incubated at 37° for 3 hr and surviving virus was estimated by plaque assay in a suspension culture of chick embryo cells (Bradish, Allner and Maber, unpublished). The $\log_{10}$ reciprocal of the dilution of serum calculated to neutralise 50 per cent of the virus was taken as the neutralising titre.

Mice.—Male Porton mice weighing 15 g. were inoculated into the left hind footpad with 0·03 ml. inocula containing an estimated 100 mouse i.c. LD_{50} SFV. Inocula of 0·5 ml. antiserum were given i.p. Mice were killed with chloroform. Serum was obtained from heart's blood and the brains and cervical spinal cords were removed. Half of each brain was set aside for virus assay and the other half was examined histologically.

Histology.—Half brains were fixed in formol saline for 1–2 weeks. Paraffin sections were cut at 7 μ thickness and stained with haematoxylin and eosin and Luxol fast blue. Frozen sections were also cut after 7 days fixation and stained by Cajal's gold chloride and sublimate method for demonstration of astrocytes.

RESULTS

Groups of mice which were treated with antiserum or diluted antiserum at intervals before or after inoculation with SFV were observed for 7 weeks. As shown in Table I, in mice that received only virus, the infection ran an acute

TABLE I.—*Deaths Among Mice Inoculated into the Footpad with* 100 LD_{50} *Semliki Forest Virus and Treated with Antiserum*

(Virus inoculation Day 0)

Treatment	Mortality	Day of deaths
Virus only	20/20	5–9
Normal serum day 1	10/10	5–7
Antiserum day −1	2/10	7, 17
Antiserum day 0	2/10	8, 9
Antiserum day 1	6/30	5, 6, 6, 16*, 21*, 26*
Antiserum diluted 1 : 10 day 1	9/20	4–9
Antiserum diluted 1 : 100 day 1	16/20	4–8 (15 mice) 14*
Antiserum day 2	6/10	5, 7, 7, 7, 7, 23

* Killed, showing clinical encephalitis.

course and all 20 mice died from 5–9 days after inoculation. All the mice inoculated with normal rabbit serum died within 7 days of inoculation. The mice treated with antiserum received a considerable degree of protection although some developed an acute encephalitis and died within 10 days of inoculation. At this time the remaining mice appeared healthy, but after a further delay clinical signs of encephalitis developed in some of them. Two deaths occurred 17 and 23 days after inoculation and 4 mice with clinical encephalitis were killed on the 14, 16, 21 and 26th days respectively. The brains of these 4 mice were examined histologically. All were found to have lesions of encephalitis.

In a further series of experiments mice were inoculated with SFV and treated with antiserum on the following day. In the first experiment of this series the brains of mice which fell sick within 10 days of virus inoculation were harvested; surviving mice were killed on the 10th day and all the brains were examined histologically. In another 4 experiments groups of 5 apparently healthy mice

were killed on the 10, 15, 20 and 27th days after virus inoculation. Individual sera were obtained for neutralising antibody assay and half brains were examined histologically and for the presence of virus. In addition, from the 10th day onwards mice with clinical signs of encephalitis were killed and their tissues were examined similarly. Since the numbers of mice in these 4 experiments were progressively reduced the observation period was limited to 49 days. In the 6th and 7th experiments in which clinically affected mice were harvested only, mortality was observed for 100 days, and in a similar 8th experiment mortality was observed for 200 days. The details of these experiments are summarised in Table II.

TABLE II.—*Synopsis of Experiments on Mice Inoculated with Semliki Forest Virus and Treated One Day later with Antiserum*

Mice inoculated only with virus	43
Mice inoculated with virus and antiserum	313
Apparently healthy mice killed for examination	
10 days after inoculation	42
15 days after inoculation	20
20 days after inoculation	20
27 days after inoculation	20
Clinically affected mice which died or were killed	
Within 10 days of inoculation	40
From 11 to 27 days after inoculation	28
From 28 to 100 days after inoculation	7

Of 43 mice which received no antiserum, 42 died within 10 days of virus inoculation and the remaining mouse died on the 13th day. A total of 313 mice were treated with antiserum and 40 (12 per cent) of these died or were killed with clinical signs of encephalitis within 10 days of virus inoculation. On the 11th day there were 231 mice remaining and 35 (15 per cent) of these died or were killed with encephalitis with a delayed onset. There were 6 deaths between the 11–15th days, 22 deaths between the 16–27th days and 7 deaths between the 28–100th days after inoculation. The last 3 deaths occurred on the 70, 75 and 99th days: no sickness or deaths were observed in 38 mice that were kept for a further 100 days in the 8th experiment.

EXPLANATION OF PLATES

FIG. 1.—Type A lesions; cortex showing widespread degeneration and necrosis of neurones, moderate spongy degeneration, and absence of perivascular inflammatory changes. H. and E. ×160.

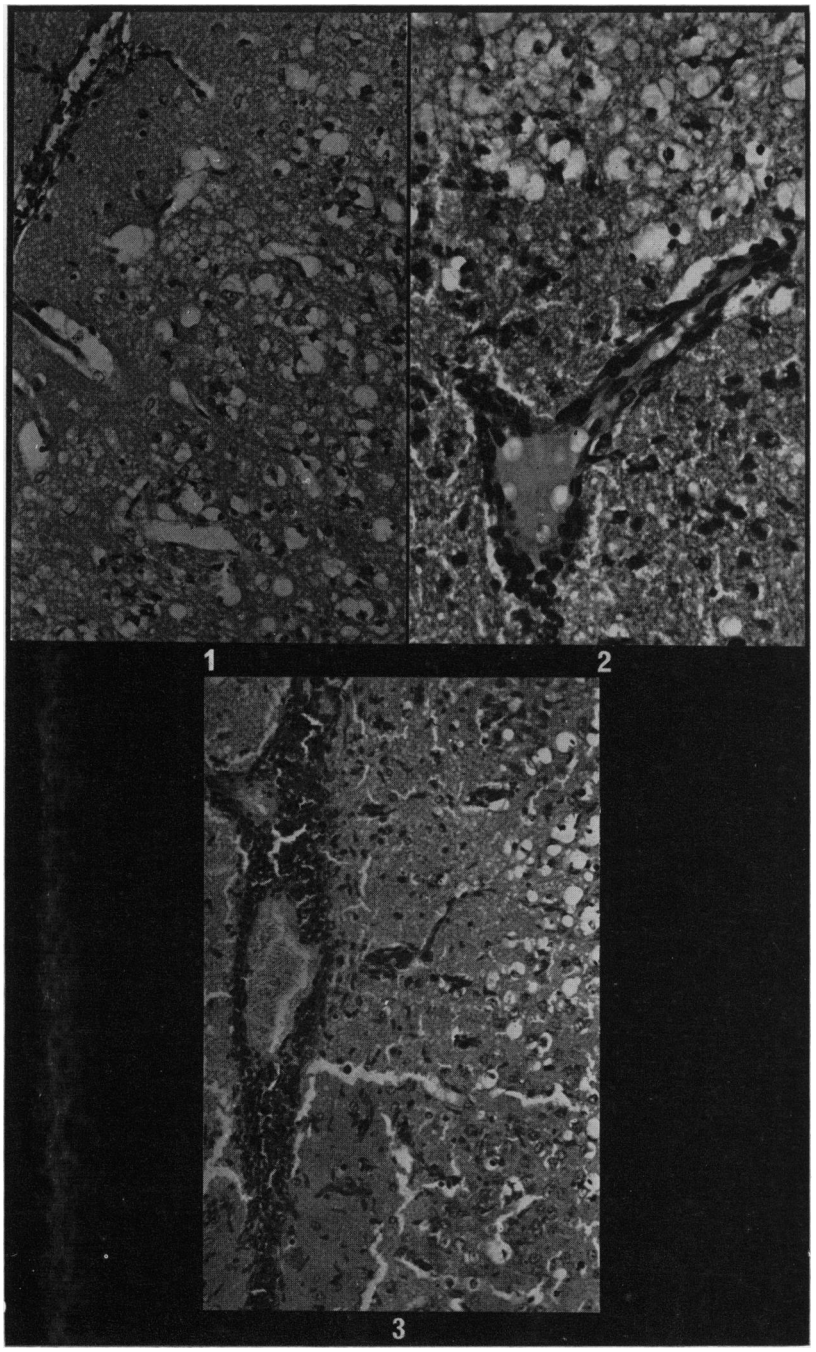
FIG. 2.—Type A lesion: thalamus showing very severe spongy degeneration, necrosis of almost all neurones and a perivascular reaction consisting of 1 layer of infiltrating cells. H. and E. ×250.

FIG. 3.—Type B lesion; cortex showing widespread perivascular infiltrations in the leptomeninges, microglial transformation and moderate neuronal and spongy degeneration. H. and E. ×160.

FIG. 4.—Type C lesion; cerebellar peduncle showing large perivascular cuffing surrounded by a zone of very severe spongy degeneration and widespread microglial infiltrations. H. and E. ×185.

FIG. 5.—Type D lesion; cerebellum showing a very large perivascular cuffing surrounded by a zone of microgliosis. (Note absence of degeneration in Purkinje and granule cells). H. and E. ×185.

FIG. 6.—Type E lesion; residual changes in the gyrus subcallosus consisting of small infiltrations in the leptomeninges and 2 microglial infiltrations in the adjoining brain substance. H. and E. ×185.



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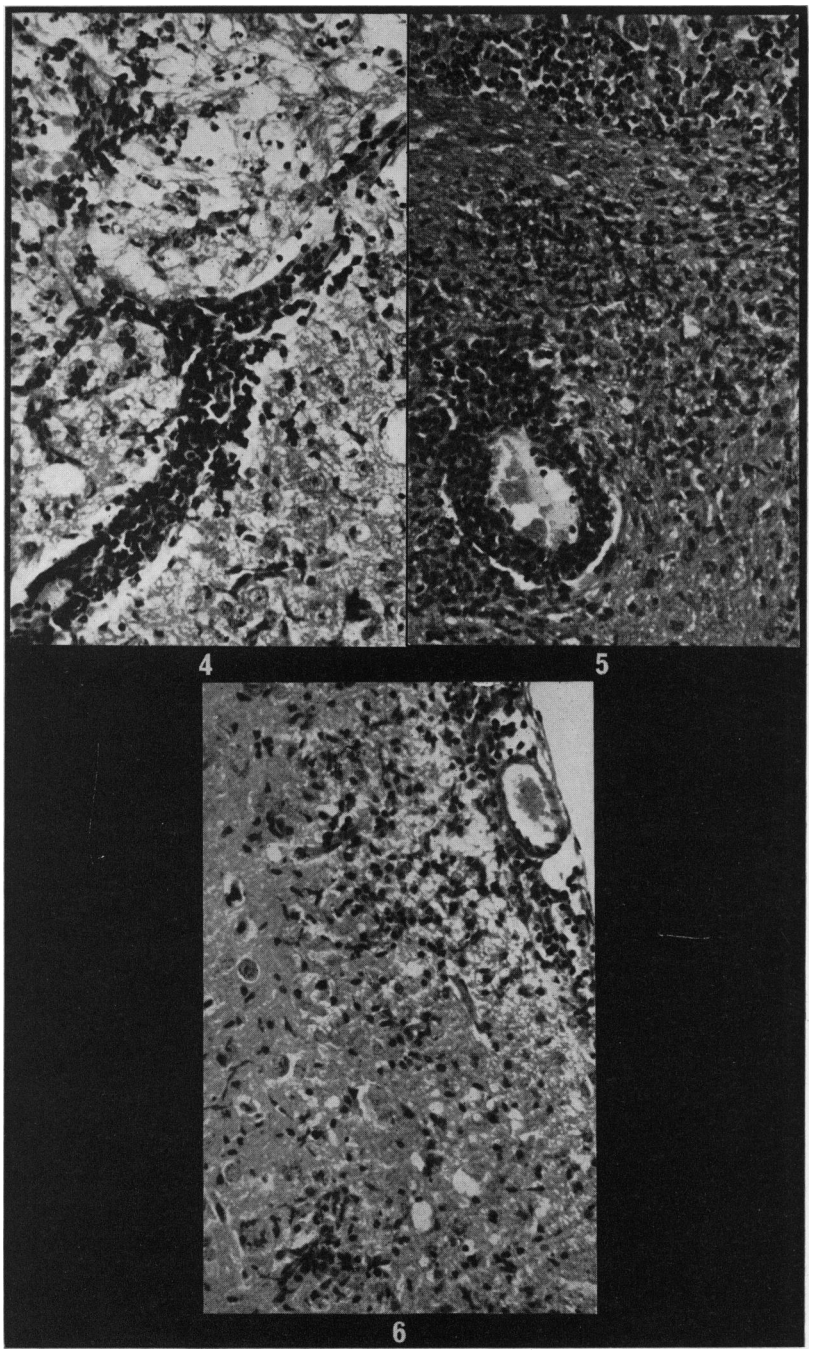

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Examination of mice with clinical signs

As shown in Table III, 21 clinically affected mice were examined. High virus titres ranging from $10^{6\cdot1}$–$10^{9\cdot5}$ pfu per g. were found in the brains of 12 mice that developed clinical signs between 14–70 days after inoculation with virus. No virus was detected in the brains of 6 other clinically affected mice.

TABLE III.—*Examinations of Mice with Clinical Encephalitis after Inoculation with Semliki Forest Virus and Treatment with Antiserum*

Mouse No.	Days after virus inoculation	Brain virus titre $\log_{10}$ pfu/g.	Serum neutralising titre	Type of brain lesion*
1	5	NE	NE	B
2	5	NE	NE	A
3	5	NE	NE	A
4	14	7·5	2·3	B
5	16	nil	2·0	C
6	17	8·5	2·3	B
7	17	6·1	2·6	B
8	20	7·8	3·3	B
9	20	nil	4·0	C
10	21	9·1	2·8	A
11	22	7·7	3·6	B
12	24	9·2	>2·0	A
13	24	7·7	2·0	B
14	24	nil	2·0	D
15	24	nil	2·0	D
16	26	8·4	4·4	B
17	31	8·8	2·3	B
18	36	8·0	4·1	B
19	39	nil	<2·0	D
20	70	9·3	2·3	B
21	75	nil	2·5	C

* Descriptions in text.
NE = not examined.
nil = no virus detected in 10 per cent brain suspension.

A wide range of neutralising antibody titres was found in the sera of clinically affected mice (Table III). Most titres tended to be low, but very high titres were found in 3 mice (Nos. 9, 16 and 18).

Pathological changes seen in the brains of clinically affected mice ranged from acute inflammatory and often necrotising lesions to those more subacute and predominantly allergic in character in which huge perivascular cuffings and infiltrations were prominent. Four types of lesion were found:

Type A was commonly found in mice inoculated only with virus and in mice that developed signs within a few days of inoculation and treatment with antiserum (Table III). The lesions were usually more numerous and more marked in the cerebral cortex, corpus striatum and hippocampus than in any other parts, and as a rule were mild in the cerebellum and brain stem. The pathological process consisted of a diffuse meningo-encephalitis with a scanty meningeal cellular exudate and vascular changes restricted either to swelling of the capillary epithelium or to 1 layer of infiltrating cells resembling microglia around the blood vessel. Occasionally small lymphocytes were also present in the perivascular exudate. The brain parenchyma had many microglial accumulations with numerous cells undergoing transformation into stab cells. This was especially noticeable in the pyriform cortex and hippocampus in the form of severe neurono-

phagia of neurones undergoing eosinophilic degeneration. In moribund mice the most noticeable lesion apart from widespread haemorrhages was widespread necrosis of neurones and spongy degeneration. In some cases large areas of cortex, hippocampus and thalamus were transformed into a loose network of fibres with scattered necrotic neurones in the meshes (Fig. 1 and 2).

Type B lesions were seldom found in mice that received only virus, but were common in mice treated with antiserum. The lesions were very widespread throughout the brain including the cortical and subcortical centres. They were either focal or diffuse and resembled type A lesions but many perivascular cuffings were composed of several layers of lymphocytes and were often surrounded by a zone of transformed microglial cells. Spongy degeneration and necrosis of neurones were also common in advanced clinical cases (Fig. 3).

Type C lesions were found only in mice treated with antiserum (Table III). They consisted of subacute, often huge, perivascular cuffings composed of several layers of lymphocytes surrounded either by circumscribed zones of very severe spongy degeneration with infiltrations of small microglial cells or by diffuse necrosis of all elements. These foci were present throughout the brain but were more numerous and much more severe in the brain stem, mid brain and cerebellum. As a rule all the neurones in the centres of spongy degeneration were undergoing degeneration or necrosis (Fig. 4).

Type D lesions were found in mice that developed disease a relatively long time after treatment with antiserum (Table III) and were confined to the cerebellum and brain stem. They consisted of perivascular cuffings with several layers of lymphocytes occasionally surrounded by a zone of microglial cells. In the affected areas small microglial foci and neuronal degeneration were occasionally present (Fig. 5).

Examination of infected mice which showed no signs of disease

As shown in Table II groups of at least 20 mice that showed no clinical signs were killed for examination on each of the 10, 15, 20 and 27th days after inoculation with virus. No virus was detected in 10 per cent suspensions of half brains harvested from 15 mice on each of these days (Table IV). At these intervals the numbers of mice with serum neutralising antibody titres exceeding 2 were 18/20, 18/20, 15/20 and 13/20 respectively. The actual titres of 39 of these sera were determined and plotted against time to give an estimate of the half life of the antibody. A value of 5·3 days was obtained with 95 per cent fiducial limits of

TABLE IV.—*Examinations of Mice Which Showed no Signs of Disease after Inoculation wtih Semliki Forest Virus and Treatment with Antiserum*

	Days after virus inoculation			
	10	15	20	27
Virus in brain	0/15	0/15	0/15	0/15
Serum neutralising antibody titre > 2	18/20	18/20	15/20	13/20
Brain lesions Type D*	1	—	1	—
Type E*	5	2	2	1
No. lesions	36	18	17	19
Total examined	42	20	20	20

* Descriptions in text.

3·3 and 13·6 days. The half life of SFV antibody as determined in 50 normal mice was found to be 5 days with 95 per cent fiducial limits of 4·3 and 6·0 days.

The brains of 102 apparently healthy mice were examined histologically. No lesions were found in 90 of these brains. Type D lesions (see preceding section) were found in the brain of 1 mouse killed 10 days after virus inoculation and in another killed 20 days after inoculation. In the remaining 10 mice, lesions of Type E were found: these could be regarded as residual changes after recovery from encephalitis. They were found scattered throughout the brain and consisted of swelling of the capillary endothelium, small glial knots, perivascular rosettes, incomplete perivascular infiltrations with darkly stained cells. Some foci of neuronephagia were present but there was no evidence of neuronal necrosis. Small circumscribed areas of cellular infiltrations in the leptomeninges were also found in some brains (Fig. 6).

DISCUSSION

The administration of antiserum failed to protect some mice infected with SFV and these developed acute encephalitis and died at the same time as untreated mice. On the other hand, a large number of mice treated with antiserum survived for the whole of the observation period and these appeared to have been protected completely. It is reasonable to suppose that the numbers of mice which died acutely or those which were protected completely could have been varied by altering the timing or the dosage of the antiserum treatment: however the results in these mice are interesting only in the light they shed upon mice which survived the early stage of infection but developed encephalitis and died several weeks after inoculation of virus.

No lesions were found in 90 (90 per cent) of 102 apparently healthy mice killed 10–27 days after inoculation, and no virus was detected in 60 such brains. This strongly implies that no infection of the brain occurred in these mice. Additionally the estimated half life of SFV antibody (5·3 days) was essentially the same in these mice as in passively immunised normal mice (5·0 days). This also suggests that inoculation of virus led only to a very limited degree of infection since it did not give rise to the production of additional antibody, and the possibility must be considered that the administration of antiserum may in some way have desensitized the antibody producing system of the mouse. However the wide fiducial limits obtained for the antibody half life in infected mice may be interpreted as suggesting that in some mice at least the infection was sufficient to stimulate antibody production and to result in active immunity. This is supported by the finding of lesions in 12 of the 102 brains examined. The lesions in 10 brains were of a residual nature (type E) and were therefore suggestive of a past or subacute infection. These mice might be expected to have a degree of resistance to further infection with SFV.

Delayed encephalitis occurred in 35 of the 231 mice (15 per cent) that remained on the 11th day after virus inoculation. Since a further 60 mice were killed on or before the 27th day, the 15 per cent which developed delayed encephalitis can be regarded as a minimal figure. Very high titres of virus were found in 12 of the 18 brains taken from mice with delayed encephalitis, but no virus was detected in 6. In mice with high virus titres acute brain lesions of types A and B were also present, but lesions of a more subacute or allergic nature (types C and D) were found in mice in which no virus was detected. There appears to be no simple

relationship between the neutralising antibody titres found in mice with delayed encephalitis and the presence of lesions or virus in the brain (Table III). However, since the half life of antibody in normal mice was found to be 5 days, it would appear that the serum neutralising titres found in some mice with encephalitis 10 to 20 days after inoculation were rather low and that the titres of those examined after 30 days were higher than would be expected with the decay of passively administered antibody.

The appearance of SFV so long after the original infection is explicable only in terms of persistence. A group B arbovirus, that of Kyasanur Forest Disease, was isolated by Price (1966) from the brains and livers of mice for up to 246 days, but these mice were chronically diseased and the virus was probably present almost continuously in detectable levels. Since SFV was not detected in the brains of apparently healthy mice 10 to 27 days after inoculation, the virus either persisted in a latent form at levels below the threshold of the methods used for detection, or it was present in other organs. It seems probable that in some mice such a prolonged infection flared up and overwhelmed the animals with an acute disease: this would be most likely to occur in mice which had not become actively immune. (In these mice the administration of immune serum can be compared to immunosuppression since the development of active immunity is prevented, however, unlike immunosuppression the mouse is protected against the degenerative and necrotising effects of the virus).

It is difficult to advance a satisfactory explanation of the pathogenesis of the encephalitis in mice in whose brains no virus was detected. Failure to demonstrate virus in these brains does not necessarily mean that virus was not present. If virus persisted at low levels in mice which had developed a degree of immunity, any resurgence or virus would be checked, but the subacute or allergic type of lesion found in some brains could be expected. These deaths are thus comparable in some ways to the delayed deaths produced by the administration of antiserum to mice infected with Langat virus (Webb *et al.*, 1968) but these workers did not examine the brains of their mice for the presence of virus. The delayed development of encephalitis without high levels of virus multiplication is also somewhat similar to subacute sclerosing panencephalitis in man (Zeman and Kolar, 1968; Lennette, Magoffin and Freeman, 1968) and the SFV-antiserum system in mice may prove to be a useful model for investigations into this human disease.

We are grateful to Mr. S. Peto for statistical assistance and to Mr. H. B. Maber and Mrs. P. Stenhouse for skilful technical assistance.

REFERENCES

Berge, T. O., Gleiser, C. A., Gochenour, W. S. Jr., Miesse, M. L. and Tigertt, W. D.—(1961) *J. Immun.*, **87**, 509.

Lennette, E. H., Magoffin, R. L. and Freeman, J. M.—(1968) *Neurology*, **18**, 21.

Price, W. H.—(1966) *Virology*, **29**, 679.

Seamer, J., Randles, W. J. and Fitzgeorge, R.—(1967) *Br. J. exp. Path.*, **48**, 395.

Smithburn, K. C. and Haddow, A. J.—(1944) *J. Immun.*, **49**, 141.

Webb, H. E., Wight, D. G. D., Platt, G. S. and Smith, C. E. G.—(1968) *J. Hyg., Camb.*, **66**, 343.

Zeman, W. and Kolar, O.—(1968) *Neurology*, **18**, 1.

Zlotnik, I., Smith, C. E. G., Grant, D. P. and Peacock, S.—(1970) *Br. J. exp. Path.*, **51**, 434.