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Langat virus encephalitis in mice

I. The effect of the administration of specific antiserum

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Webb & Smith (1966) suggested that the clinical manifestations and histological abnormalities of virus diseases of the central nervous system (CNS) may be due not only to nerve cell destruction by virus multiplication but also to some kind of virus (antigen)-antibody reaction. The main reasons for this hypothesis were: (*a*) that the CNS disturbance almost invariably takes place after the end of the viraemia when antibody is increasing in the blood; (*b*) that only a very small percentage of all those infected actually develop clinical encephalitis, suggesting some individual response by the infected person which predisposes towards CNS involvement; and (*c*) that in immune tolerance experiments with lymphocytic choriomeningitis virus (Weigand & Hotchin, 1961) which invades the CNS, no disturbance occurs when an immunological response is absent.

The experiments reported here show that although administration of antibody before infection can protect mice from encephalitis, antibody given on certain days after infection can aggravate the disease. A second paper describes experiments which show how depression of the immunological mechanisms by irradiation affects the development of encephalitis in infected mice. Langat virus is particularly suitable for these experiments as it causes clinical encephalitis in only a small proportion of mice if given intraperitoneally (i.p.); thus it simulates to some extent the behaviour of similar viruses in humans.

MATERIALS AND METHODS

Langat virus

The TP21 strain of Langat virus was used in its seventh mouse passage as a 10% mouse brain suspension in bovine albumen phosphate saline (BAPS). Virus titrations were performed by diluting blood or mouse brain suspensions in 10-fold steps in BAPS and inoculating volumes of 0·03 ml. intracerebrally (i.c.) or 0·1–0·2 ml. i.p. in mice 3–4 weeks old. Blood samples for viraemia estimations were obtained from the orbital plexus using heparin as an anticoagulant. In several experiments half a brain was preserved for histological examination and half titrated for virus. Virus titres were calculated by the method of Reed & Muench (1938).

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Mice

The mice used were of the Swiss A_2G strain of either sex, received from the Glaxo Research Farm, Harefield, Middlesex.

Antibody preparations

Human ascitic fluid samples were taken before and after the infections from a patient with carcinoma of the ovary who had been treated first with Langat virus and then with the closely related Kyasanur Forest disease virus (Webb, Wetherley-Mein, Smith & McMahon, 1966). The post-infection ascitic fluid used in these experiments was taken following the second infection. This fluid had a neutralizing index of at least 1000 and a haemagglutinin-inhibiting (HI) titre of 1/320 to Langat virus. Other human sera taken before and after Langat infections were used with similar results.

Swiss A_2G mouse serum was obtained before and after repeated i.p. inoculations of Langat virus. This serum had a neutralizing index of 630 and an HI titre of 1/40. All antibody preparations used were bacteriologically sterile.

Experimental

In the experiments the virus was given i.p. in 0·1–0·2 ml. quantities in doses ranging from 50 to 1,000,000 ICLD 50. The antibody preparations were given in doses of 0·1–0·2 ml. i.p., 0·1 ml. intravenously (i.v.) into the tail vein or 0·03 ml. i.c. For intracerebral or intravenous inoculation ether anaesthesia was used. In every experiment where an antibody preparation was given, a control group of mice was treated similarly with comparable material without antibody. None of the fluids were toxic to mice when inoculated alone. All mice were observed for at least 3 weeks and often for 4 weeks. In experiments where a sufficient number of mice survived, evidence of infection in them was established either by testing their sera for HI antibody, or (as this titre was low in many mice previously given anti-serum early in the viraemia) they were challenged i.c. with 100–1000 ICLD 50 of virus. Paralysis rates rather than mortality rates are reported in the Tables because some paralysed mice survived to the end of the observation period. Most mice, however, had died by the twenty-first day.

Antibody studies

Haemagglutinin-inhibiting antibody levels were determined by the method of Clarke & Casals (1958) as modified by Sever (1962) and others. The heparin contained in the mouse plasmas was found to cause non-specific haemagglutinin-inhibition against Semliki Forest virus but not Langat virus. It was removed by treatment with protamine sulphate during acetone extraction (Holden, Muth & Skinner, 1966) without loss of antibody. The plasma extracts were tested against eight haemagglutinin units of Langat virus and of Semliki Forest virus as a control.

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Histology

Brains and spinal cords were fixed in 10% formol saline and embedded in paraffin wax. Sections of at least five levels of the brain and three levels of the spinal cord were cut at 5 μ and stained with haematoxylin and eosin. All brains were fresh when fixed and any mice found dead were discarded. The distribution and severity of lesions seen in brain sections were graded as follows (modified after Berge *et al*. 1961):

0 (negative): No evidence of perivascular cuffing or increase in glial cells, normal mouse brain.

1: Mild perivascular cuffing and/or slight focal increase in glial cells.

2: Definite perivascular cuffing without distension of the Virchow–Robin spaces by multilayered cells and/or definite glial nodules without disturbance of histological architecture.

3: Marked perivascular cuffing and moderate increase in cellularity of glial nodules, with in addition more diffuse glial proliferation. Slight disturbance of background architecture and minimal neuronal damage present.

4: Marked perivascular cuffing with considerable proliferation of glial elements with alteration of ground substance often with vacuolization. Obvious neuronal destruction present.

RESULTS

Infections without administration of antibody

Many preliminary control experiments were carried out in which mice were killed on various days after infection either i.c. and i.p., and their brains and spinal cords examined histologically.

Intracerebral inoculation

There was a diffuse encephalomyelitis with a fairly rapid progression from the appearance of the first lesions as early as 5 days after infection until death on the seventh to ninth day. There was well defined mononuclear cell infiltration of the leptomeninges and of the perivascular spaces with a variable amount of migration of these cells into the surrounding brain substance. There were areas where proliferation of all the neuroglial elements, astrocytes, oligodendroglia and microglia, had occurred. These changes were seen at all levels of the brain and spinal cord. Neuronal changes, which were prominent in those mice which were clinically paralysed and ill, consisted of a patchy necrosis of all layers of the cerebral cortex of the hippocampus and caudate nucleus-putamen. The majority of the affected cells were shrunken and eosinophilic with pyknosis or karyorrhexis of the nuclei. Degeneration of the Purkinje cells of the cerebellum and of the ventral horn cells of the spinal cord was sometimes seen. The majority of the mice which were ill when killed had lesions of grade 4.

Intraperitoneal inoculation

The encephalomyelitis when this did occur was essentially similar. However, the progression of lesions was slower, the first changes were seen as early as the

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fifth day, yet advanced encephalomyelitis was often seen as late as the fifteenth day. There was also much greater variability in the intensity of the lesions. Although lesions were seen in all mice, in many these consisted only of scattered glial nodules and perivascular cuffs. The lesions in the most severely affected brains were of a characteristic distribution: the olfactory lobes and tracts, the cerebral cortex, the corpus striatum, the cerebellum and the grey matter of the spinal cord were constantly involved but the lesions were much less diffuse than following i.c. inoculation

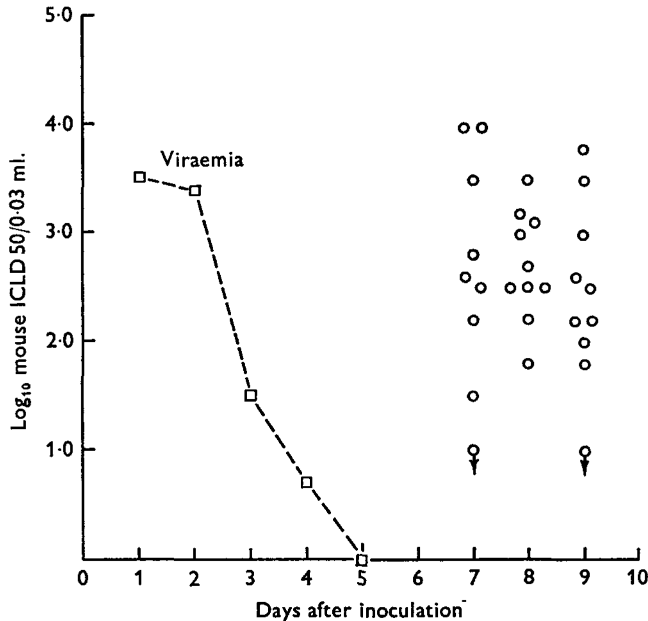

Fig. 1. Viraemia and virus titres in brain after intraperitoneal inoculation of 10,000 ICLD 50 of Langat virus on day 0. □ Viraemia (four bloods pooled each day). ○ Individual brain virus titres on days 7, 8 and 9.

Infections with administration of antibody

These experiments were designed to demonstrate the effect of antibody given 1–2 hr. prior to inoculation of virus, and on each post-inoculation day up to the sixth, which was the day before the first mice showed paralysis. As many as fifty mice were used in each group in some experiments so that both survival and histological studies could be done simultaneously. There were never less than eighteen mice in any group. Figure 1 shows the viraemia pattern in mice following i.p. infection. Mice with no evidence of clinical encephalitis were found nevertheless to have both histological encephalitis and high brain virus titres. In this experiment only nine of the thirty mice observed for symptoms had an encephalitic illness, and five of them died. However, all the brains of the similarly inoculated mice taken on days 7, 8 and 9, had incontrovertible evidence of encephalitis and all except two showed more than ten ICLD 50 virus per 0·03 ml. of a 10% suspension. This was at a time when viraemia had ceased at least 48 hr. earlier. The

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higher the virus dose the earlier the viraemia started. Older mice tended to have viraemia a little later than the younger mice, but viraemia had ceased before the sixth day in all.

Table 1. *Comparison of the viraemia following i.p. inoculation with* 10,400 *ICLD* 50 *of Langat virus in a control group and in experimental groups, one of which was given* 0·2 *ml. i.p. of a* 1/4 *dilution of pre- or post-infection human ascitic fluid on each of the first* 5 *days after inoculation*

Pre-infection ascitic fluid given on post-inoculation day	Virus titre in blood on post-inoculation day						
	1	2	3	4	5	6	7
1	.	2·5*	1·5	0·8	1·3	0·3	+
2	.	.	1·5	1·1	1·5	0	0
3	.	.	.	1·5	1·5	+	0
4	.	.	.	.	0·7	0	0
5	.	.	.	.	.	+	0
Controls for both groups	2·0	2·2	2·0	1·2	1·3	0	0
Post-infection ascitic fluid given on post-inoculation day							
1	.	+	+	0	0	0	0
2	.	.	0	0	+	+	0
3	.	.	.	+	+	0	0
4	.	.	.	.	0	0	0
5	.	.	.	.	.	0	0

* Virus titres measured in $\log_{10}$ mouse ICLD 50/0·03 ml.
\+ = Virus just detectable when neat blood was inoculated.
0 = No virus detectable when neat blood was inoculated.

Table 2. *Comparison of the effects of* 0·1 *ml. of undiluted pre- and post-infection human ascitic fluid given i.p. to a group of* 4–6 *weeks old mice on each of the first* 6 *days after infection i.p. with* 400,000 *ICLD* 50 *of Langat virus*

Ascitic fluid given	Proportion of mice paralysed after	
	Post-infection ascitic fluid	Pre-infection ascitic fluid
Before infection	0/25	7/25 (28)
(Days after)		
1	16/24 (67)	9/24 (37)
2	17/24 (71)	5/24 (21)
3	9/24 (37)	8/24 (33)
4	8/24 (33)	5/24 (21)
5	2/24 (8)	5/24 (21)
6	4/24 (17)	6/24 (25)

Figures in parentheses indicate percentages.

Table 1 shows the viraemia of mice after administration of a 1 in 4 dilution of pre- or post-infection human ascitic fluid to a group of mice on each day after inoculation of virus. A control group given virus only was included. On each day, two mice were bled and killed, their bloods pooled and titrated for virus, and their

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brains kept for histology. The pre-infection fluid had no significant effect on viraemia. The post-infection fluid quite clearly depressed the viraemia, though a trace was detectable occasionally. Histological lesions were first seen on the sixth day in all groups. There was a marginal reduction in the histological evidence of encephalitis when post-infection fluid was administered on days 1–4 as compared to pre-infection fluid. When post-infection fluid was administered on the fifth day, a slight increase in the number of lesions was seen as compared to mice given pre-infection fluid.

Table 2 shows the paralysis rates when human post-infection fluid was given i.p. on the specified days compared to pre-infection fluid controls. Here, the post-infection fluid clearly provided complete protection when it was given before virus inoculation, whereas, when it was given on either of the first 2 days, paralysis was significantly more frequent.

Table 3. *Comparison of the effects of* 0·1 *ml. of undiluted pre- and post-infection human ascitic fluid given i.v. to a group of* 4–6 *weeks old mice on each of the first* 5 *days after infection i.p. with* 400,000 *ICLD* 50 *of Langat virus*

Ascitic fluid given after infection (day)	Proportion of mice paralysed after	
	Post-infection ascitic fluid	Pre-infection ascitic fluid
1	9/19 (47)	2/18 (11)
2	13/20 (65)	5/20 (25)
3	9/20 (45)	6/20 (30)
4	2/20 (10)	4/20 (20)
5	1/20 (5)	1/20 (5)

Figures in parentheses indicate percentages.

Table 4. *Comparison of the effects of* 0·1 *ml. of pre- and post-infection human ascitic fluid, normal mouse serum and mouse antiserum given i.v. to* 6–8 *weeks old mice on the third day after infection i.p. with* 400,000 *ICLD* 50 *of Langat virus*

Proportion of mice with paralysis after			
Post-infection ascitic fluid	Pre-infection ascitic fluid	Mouse antiserum	Normal mouse serum
17/28 (61)	4/29 (14)	14/23 (61)	2/24 (8)

Figures in parentheses indicate percentages.

Table 3 shows the paralysis rate in the mice when human post-infection fluid was given intravenously on the specified days compared to pre-infection controls. Once again, a significantly increased paralysis rate occurred when antibody was given 1 or 2 days after virus. Both these Tables suggest a decreased paralysis rate when post-infection fluid is given particularly on days 5–6 (after the height of the viraemia). The possible reasons for this are discussed at the end of the following paper.

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Table 4 compares the paralysis rates when mouse normal serum or antiserum, or human pre- or post-infection ascitic fluid was given i.p. on the third day. This shows that the difference in paralysis rate when antibody is given on the third day can be as great as seen in the previous experiments when it was given on the first 2 days.

In all these experiments paralysis in control mice and in those given pre-infection fluid was first observed between the sixth and thirteenth day after infection (most commonly on the eighth day), whereas in the mice given post-infection fluid on any day up to the fifth day, the earliest onset of paralysis was on the thirteenth day

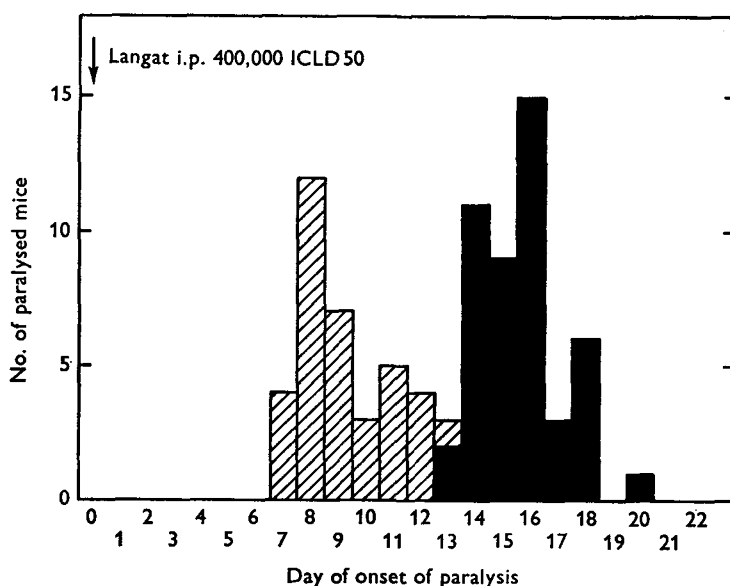

Fig. 2. Day of onset of paralysis after intraperitoneal inoculation of 400,000 ICLD 50 of Langat virus only (controls) and after pre- and post-infection ascitic fluid on days 1–3. ▨ Mice receiving pre-infection ascitic fluid and control mice. ■ Mice receiving post-infection ascitic fluid.

after infection, but could be delayed to the twentieth day (most commonly on the sixteenth day) (Fig. 2). Thus, the antiserum delayed the onset of paralysis by about 7 days, and increased the proportion affected compared to those mice given virus only and mice given virus followed by normal serum.

In the experiment reported on Table 4, up to four mice were taken for histology from each of the four groups (human post- and pre-infection fluid and mouse antiserum and normal serum) on at least 3 days between the seventh and fourteenth days after infection. Eight mice from the group which had received mouse antiserum were taken after the onset of clinical paralysis 17–21 days after infection, each being matched with a mouse from the non-immune group (none of which showed evidence of paralysis). Finally, ten clinically healthy mice were taken from each group 21–28 days after infection, and after bleeding were killed for histo-

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logical examination. There were separate groups for histological examination quite distinct from those for observation. The results fall into three categories:

Category 1. *Mice examined before paralysis* (7–14 *days*)

Three or four mice were selected at random from each group on each day, thus some of these mice would have subsequently become paralysed had they been permitted to survive. Table 5 shows that there is a clear difference between the groups which received human post- and pre-infection ascitic fluid: in the latter group mild lesions were first seen in 2/4 mice on the seventh day, and there was a steady increase in intensity of the lesions up to the fourteenth day, all mice being affected; in contrast, only one of the former group on the seventh or ninth day showed any evidence of encephalitis (graded 1) and only 2/4 and 2/3 taken on the eleventh and fourteenth days respectively were affected.

Table 5. *Comparative histology in groups of four mice given* 0·1 *ml. i.v. of pre- or post-infection ascitic fluid* 3 *days after infection i.p. with* 400,000 *ICLD* 50 *of Langat virus, and killed* 7–14 *days after infection*

Days after infection with virus	Human ascitic fluid: Post-infection				Human ascitic fluid: Pre-infection			
7	0	0	0	1	0	0	1	1
9	0	0	0	0	1	1	1	2
11	0	0	1	4	1	2	3	3
14	0	2	4	—	3	3	3	4

No comparable histological differences in time of onset were found between the two groups which had received mouse serum. However, the mouse antiserum had a much lower titre than the human post-infection ascitic fluid used.

Category 2. *Mice examined after the onset of paralysis* (17–21 *days*)

These were all taken from the group which had received mouse antiserum and were compared with mice which had received normal mouse serum and were not paralysed. All the paralysed mice showed extensive changes (graded 4, one 3): particularly in the hind brain and spinal cord, whereas all the non-immune group showed mild changes (1 or 2, one mouse 3). This latter finding was unremarkable since there were no new cases of illness as late as the seventeenth day in the mice in this group which were kept for observation and thus severe changes would not be expected. Consequently, it was decided to compare the histological changes in the paralysed group with those in mice from previous experiments which had received a comparable dose of virus and which had been paralysed for a similar period before being killed. They had received no serum or treatment of any kind and had become paralysed 7–10 days after infection. There was no obvious difference between the different members of this group, and all had lesions of grade 3 or 4, maximal in the spinal cord. There were, however, several differences in the

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distribution and emphasis of lesions between this control group and the paralysed group which had received antiserum, although the overall grades were closely similar in both groups. Lesions in the cerebellum were more frequent and intense in the group which had received antiserum; in particular there was more perivascular cuffing in the central white matter, and the thickness of the cuffs tended to be greater. Lesions were also of greater intensity in the spinal cord with, again, a slight increase in number and thickness of perivascular cuffs, together with an increase in the amount of reactive glial hyperplasia and neuronophagia. In contrast there were fewer lesions elsewhere in the CNS than in the control group. Mice given human ascitic fluid were not examined at this stage.

Table 6. *The comparative histology and HI titre in mice given pre- and post-infection human ascitic fluid and surviving* 21 *days*

	Human ascitic fluid			
	Post-infection		Pre-infection	
Day after inoculation	Grade	HI	Grade	HI
21	0	< 10	1	40
	0	< 10	2	⩾ 160
	2	20	3	80
	2	40	2	80
	0	< 10	1	80
	2	10	2	40
22	0	< 10	2	⩾ 160
	3	20	2	80
	2	10	1	⩾ 160
	1	< 10	2	⩾ 160
	2	20	1	80
23	2*	20	1	⩾ 160
	3*	20	3	⩾ 160
	2*	20	1	80
	1*	10	1	⩾ 160

* = Paralysed mice.

Category 3. *Mice taken* 21–28 *days after infection*

These mice were selected because at this time new cases of illness had ceased to appear in the observation groups. In the human ascitic fluid groups (Table 6) four of the fifteen mice which had received post-infection fluid escaped lesions as compared to none of the twenty which received pre-infection fluid. Apart from this there were no obvious differences histologically between these groups. All these mice were bled before they were killed and their HI antibody was measured. The values were significantly lower (geometric mean eight-fold lower) in the mice which had been given human post-infection fluid. Notably four of the five mice without HI antibody were the four mice without histological lesions. This may have been due to failure to infect. It is also of interest that the four mice killed on the twenty-third day were all paralysed and it can be seen that they too have HI anti-

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body titres in the lower range. None of these distinctions were observed in the groups which had been given mouse serum.

Table 7 shows the average time of death and paralysis rate of mice given virus i.p. and post- or pre-infection ascitic fluid i.c. on the specified days. Even 0·03 ml. of post-infection fluid given i.c. before inoculation of virus completely protected the mice and, in contrast to the previous experiments, intracerebral post-infection fluid on the first three post-inoculation days also provided some immunity. Furthermore there was a marked increase in death rate compared with the other experiments with pre-infection fluid. This was almost certainly due to the procedures of needling of the brain and thereby introducing virus directly into it. Our experience has shown that i.c. inoculation given just before or at the time of the viraemia greatly increases the mortality of the mice: not immediately following the inoculation but as a very greatly increased rate of paralysis and death at the normal time expected. It seems probable that the protection produced by inoculating the brain with antiserum at the height of the viraemia is because the secondary virus cycle in the brain is suppressed.

Table 7. *Comparison of the effects of* 0·03 *ml. of undiluted pre- and post-infection human ascitic fluid given i.c. to groups of twenty-five mice before and on each of the first* 5 *days after infection i.p. with approximately* 400,000 *ICLD* 50 *of Langat virus*

	Post-infection ascitic fluid		Pre-infection ascitic fluid	
Serum given	ADD*	Paralysed	ADD	Paralysed
Before infection		0/25	9·5	19/25 (76)
(days after)				
1	16·5	13/25 (52)	9·2	24/25 (96)
2	15·0	10/25 (40)	10·0	17/25 (68)
3	16·0	8/25 (32)	10·3	11/24 (46)
4	14·5	12/23 (52)	10·0	6/25 (24)
5	8·2	10/23 (43)	10·1	11/25 (44)

* Average day of death.
Figures in parentheses indicate percentages.

DISCUSSION

These experiments have clearly shown that when specific antiserum was given to mice on any of the first 3 days (when the viraemia would otherwise be at its highest), the viraemia was depressed, the development of antibody was depressed and possibly delayed and the incidence of encephalomyelitis was significantly increased. Thus, as postulated by Webb & Smith (1966), an immunological reaction probably plays some part in the pathogenesis of the clinical disturbance. It is also clear (Tables 2 and 7) that a small dose of specific antibody administered i.p. or i.c. before virus inoculation was sufficient to protect the animals completely even against large doses of virus. This is compatible with the accepted use of passively administered antibody in the prophylaxis of disease. The results of the experiments using human ascitic fluid cannot be explained on the basis that human anti-

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body was becoming bound to virus perhaps at the surface of a CNS cell and that the host then rejected the antigen-antibody complex (as foreign protein) together with the cell: similar results were obtained when mouse antiserum (prepared in the same strain of mice) was used. Moreover, it is well known that it is very difficult to produce experimental allergic encephalitis in mice except in especially selected strains.

Histological examination of brains and spinal cords tended to confirm the other findings without making any positive contribution towards a solution. The finding that the onset of histological encephalitis is delayed and that the intensity of lesions between the seventh and fourteenth day (category 1) was less in animals which received human antibody than in those which received pre-infection fluid tends to support the clinical and virological results over the same period. Thus, one would expect a lower viraemia and delayed host antibody production to be associated with fewer and delayed CNS lesions. The absence of a similar histological difference between the two groups which received mouse antiserum and normal serum was probably due to the lower antibody titre of the mouse serum. Nevertheless, there was still delayed onset and an increased incidence of encephalitis. The mice which had been given mouse antiserum on the third day and were killed after the onset of paralysis (days 17–22, category 2) had more severe encephalitis and myelitis than controls (given pre-infection ascitic fluid) which were clinically healthy at this stage. Comparing the histology of the paralysed mice from this group with that of paralysed mice in control groups falling sick at the usual time, the same histological gradation was found. However, in the antibody group the lesions were concentrated in the hind brain and spinal cord, with fewer in the remainder of brain. These findings are however of doubtful significance in view of the differences in the character of the two groups of mice.

Amongst the mice of category 3 which had been given human ascitic fluid (i.e. those surviving mice taken on days 22–28 after new cases of paralysis had ceased to appear in the parallel observation group) it was of interest that four of the five mice without HI antibody were the four mice without histological lesions (Table 6). The post-infection fluid appeared to have protected these mice from infection. Thus, if these were eliminated the percentage of infected mice which developed paralysis of late onset would be even higher than indicated in Table 6, i.e. 17/24 (71%).

Berge *et al.* (1961) performed similar experiments to those reported here using Venezuelan equine encephalomyelitis (VEE) virus in mice. They found that the administration of specific antiserum up to 24 hr. after virus was followed by the appearance of histological lesions at an earlier stage than in controls given normal serum. VEE virus has a shorter incubation period than Langat virus. However, these lesions remained mild and were associated with a high survival rate. The control mice which received normal serum all died and histological examination showed very severe CNS lesions just before death. Thus, our results differ from these in several ways: (*a*) VEE virus kills all mice when administered i.p. in sufficient dosage, unlike Langat virus, thus Berge *et al.* could not have found an increase in clinical disease; (*b*) they observed their mice for only 10 days, and may

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therefore have failed to observe the late onset of paralysis seen in our experiments. The following paper (Webb *et al.* 1968) contains a fuller discussion of the way in which the immunological response may affect the development of the encephalitis.

SUMMARY

1. When mice are infected intraperitoneally with Langat virus only a small proportion develop clinical encephalitis, but all mice have substantial titres of virus in the brain and also incontrovertible histological evidence of encephalitis.

2. When specific antibody is given intraperitoneally or intravenously to mice during the first 3 days after intraperitoneal infection with Langat virus, the viraemia (normally maximal during this period) is depressed, the production of antibody is depressed or delayed, and the incidence of clinical encephalomyelitis is increased significantly.

3. Specific antibody given intraperitoneally or intracerebrally before infection, protects the animals from encephalitis.

4. These findings are discussed in terms of the histology of the central nervous system of the affected mice.

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