Br. J. exp. Path. (1971) **52**, 669.

INFECTION PATTERNS IN MICE OF AN AVIRULENT AND VIRULENT STRAIN OF SEMLIKI FOREST VIRUS

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Received for publication May 13, 1971

SUMMARY.—Young mice injected i.p. with a virulent (V 13) or an avirulent (A 7) strain of Semliki Forest virus suffered a severe generalized infection over the first 2 days with prolific virus replication in the peritoneal wall and other muscle. This replication and the level of the resulting viraemia were as high for A 7 as V 13 and possibly higher. Despite this similarity in the extent of systemic infection significant replication of V 13 was detectable in the brain, the probable site of fatal damage, at 36–40 hr, 12 hr before that of A 7: and subsequently V 13 increased until death of the mice whereas A 7 declined. After intracerebral inoculation early virus replication in the brain was similar for both strains and a generalized infection ensued similar in extent to that seen after intraperitoneal injection. Within 24 hr a primary injection of the avirulent strain by either route protected mice against a subsequent injection of the virulent strain. The different patterns of brain infection occurring after intraperitoneal injection are probably due to differential interactions of the 2 virus strains with host defence mechanisms.

THE mechanisms of virus virulence are not well understood (Bang and Luttrell, 1961; Mims, 1964; Smith, 1963; Waterson, Pennington and Allan, 1967). A major reason for the lack of knowledge is the inaccuracy of comparing quantitatively the virulence of different virus strains; for this reason, only stable virus strains of the greatest possible difference in virulence should be compared in attempts to identify virulence attributes in a manner similar to that used successfully in classical bacteriology (Smith, 1968). Only in a few cases (Bang and Luttrell, 1961; Waterson *et al.*, 1967) have such strains been obtained. Recently Bradish, Allner and Maber (1971) described well established strains of Semliki Forest virus (SFV) which differed widely in virulence. Two (V 13, virulent; A 7, avirulent) have LD_{50}s for mice by i.p. injection which differ by more than 10^6. This paper describes the beginning of attempts to identify the reasons for the differences in virulence.

Following the pattern of Seamer and Randle (1967) the virus contents of the peritoneal cavities, blood, spleens and brains of mice were determined at intervals after i.p. injection of either strain of SFV. The object was to determine whether or not the virulent strain produced a more severe peritoneal invasion and systemic infection than the avirulent strain (*cf.* strains of Japanese encephalitis virus;

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Huang and Wong, 1963) thus leading to a higher viraemia and hence, possibly, to a more rapid and extensive breach of the " blood brain barrier " as suggested by Johnson and Mims (1968). During this work Murphy, Harrison and Collin (1970) reported results with SFV in mice similar to those of Johnson (1965) with Sindbis virus in mice, namely that massive replication of virus in muscle provided the reservoir for the high viraemia. This led to an investigation of the content of the 2 strains of SFV in peritoneal wall, hind leg muscle and aorta wall.

MATERIALS AND METHODS

Mice.—Porton white mice 25–30 days old were used.

Virus.—Semliki Forest virus (SFV) strains V 13 and A 7, with LD_{50}s for mice by i.p. inoculation of < 10 and $> 10^7$ pfu respectively, were described by Bradish *et al.* (1971). After mouse brain passage (V 13, 13 passages; A 7, 7 passages) the strains were passaged once in chick embryo cells. Samples (1 ml.) of the virus pool (*ca* 2×10^9 pfu/ml.) were stored at $-70°$. The virus was diluted in Parker's medium 199 containing 5 per cent v/v calf serum before inoculation. This medium was used throughout for tissue suspension. Virus infectivity was determined by plaque assays on chick embryo cells as described by Bradish *et al.*, (1971); dilutions were made in Parker's medium 199 with 10 per cent calf serum.

Inoculation.—Groups of mice were inoculated i.p. and occasionally i.c. under ether anaesthesia, with 10^4 pfu of either strain in 0·02 ml. Parker's medium containing 5 per cent v/v calf serum.

Follow up of virus infection.—Within 30 min. of inoculation and thereafter at intervals up to 4 days, pooled samples of peritoneal washings and various organs were taken as follows from 5 mice of each group killed by cervical fracture. The skin was sterilized with alcohol and 2 ml. medium were injected i.p. After light massage the cavity was opened and 1 ml. of the washings withdrawn for pooling. The chest was opened and 0·2 ml. of heart blood was pooled in sufficient medium to provide a 10 per cent suspension. Spleen and brain were removed, weighed, washing twice with medium and pooled. All samples were stored at $-70°$ until required for virus titrations. Immediately before these titrations the brains and spleens were homogenized in Griffiths tubes and made up with medium to a 10 per cent w/v suspension. The processing of samples probably did not significantly affect their virus contents (*cf.* Albrecht, 1968); when $2{\cdot}7 \times 10^7$ pfu of V 13 were added to medium, mouse blood, mouse brain and mouse spleen, and the mixtures treated as described above including freezing to $-70°$, recovered infectivities were 1·5, 2·3, 4·2, and $2{\cdot}2 \times 10^7$ pfu respectively; corresponding figures for A 7 were $4{\cdot}9 \times 10^6$ pfu recovered as 4·6, 5·7, 6·0 and 5·7 pfu respectively. Limited experiments were done with blood and tissues from individual mice to check on possible errors of interpretation due to mouse variation (Table).

In two experiments, the distribution of virus was examined in the cells and fluid of the peritoneal washings and blood taken 36 hr after inoculation. Pooled peritoneal washings collected as described above were centrifuged ($1500 \times g$ 15 min.) and the supernatant fluid and the cells (suspended in the original volume of medium) were separately titrated for infectivity as described above for peritoneal washings. Pooled blood (9 ml.) was mixed with Alsevers solution (1 ml.) and centrifuged ($1500 \times g$ 15 min.). Plasma was removed and the cell deposit was washed once with an equal quantity of medium. The " buffy coat " was floated free from underlying red cells in medium (3 ml.). Red cells were washed again in the same volume of medium and finally suspended in medium to a volume of 9 ml. Virus in plasma, buffy coat and red blood cells was assessed separately as described for blood.

In later experiments pooled samples from 5 mice of aorta walls, peritoneal wall and hind leg muscle were examined at intervals after inoculation of virus i.p. The aorta was first washed *in situ* by introducing medium (1 ml.) with a syringe into the lower part of the dorsal aorta; it was then removed, washed in medium weighed and kept at $-70°$ until required for assay of infectivity. It was then ground in a Griffiths tube made up to a 2 per cent w/v suspension with medium and clarified by centrifugation ($1500 \times g$ 15 min.) before titration. Weighed amounts of peritoneal wall and hind limb muscle were stored at $-70°$ until required for assay. The thawed tissue was washed with medium, cut up with scissors in medium to make a 10 per cent w/v suspension; for this purpose ten 3 mm. and three 8 mm. glass beads were added and the mixture homogenized on a Rotamixer (Hoak and Tucker Ltd.) for 5 min.

before clarification (1500 × g. 15 min.) and titration. In control experiments increasing the time of homogenization to 10 min. did not significantly affect the observed virus content and when $2 \cdot 56 \times 10^{6}$ pfu/ml. of V 13 and $1 \cdot 94 \times 10^{5}$ pfu/ml. of A 7 were added to normal muscle and the mixtures treated as described $1 \cdot 70 \times 10^{6}$ and $1 \cdot 50 \times 10^{5}$ pfu/ml. were recovered respectively.

Infectivities are expressed as pfu per g. of tissue, per ml. of blood and total in the peritoneal cavity per mouse. The virus contents of spleen and brain were corrected for the virus in their contained blood estimated as 17 per cent and 3 per cent of the respective tissues (Kaliss and Pressman, 1950; Albrecht, 1968).

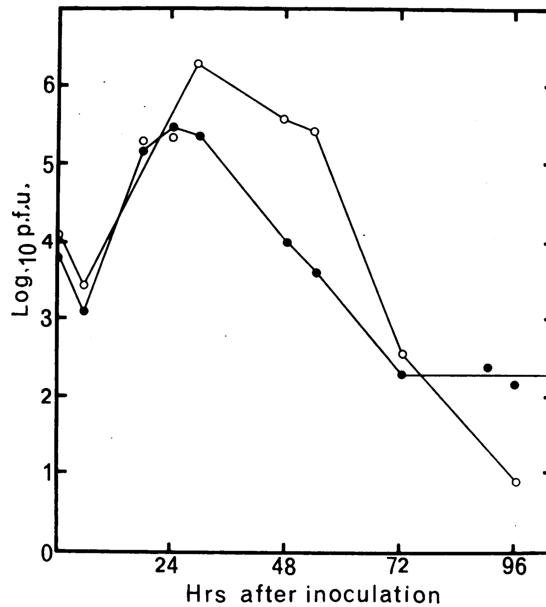

FIG. 1.—Infectivity of mouse peritoneal contents after inoculation of virulent (V 13) and avirulent (A 7) strains of SFV. ●——● V 13, ○——○ A 7. Points are obtained from pooled samples from 5 mice and represent virus in total (2 ml.) peritoneal washings from one mouse.

RESULTS

Experiments typical of a number of similar experiments are described.

Course of infection after intraperitoneal inoculation of virulent and avirulent strains of SFV

After an initial reduction, the virus contents of the peritoneal cavities reached a maximum at approximately 24 hr. for V 13 and 30 hr for A 7 and thereafter decreased rapidly; the maximum for A 7 was greater than for V 13 (Fig. 1). Both strains appeared in the blood within 30 min. and maximum infection occurred at approximately 30 hr after which it decreased rapidly; again the maximum for A 7 was greater than for V 13 (Fig. 2). The virus was not cell-borne; in both the peritoneal cavity and blood over 99 per cent of the virus was in the fluid. For both strains, there was no significant difference between the virus content of the splenic suspension and that of their contained blood until the end of the

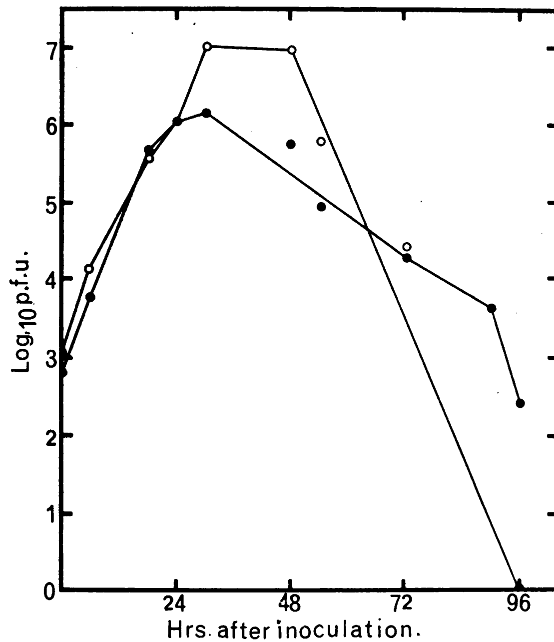

Fig. 2.—Infectivity of mouse blood (pfu/ml.) after inoculation of virulent (V 13) and avirulent (A 7) strains of SFV. ●——● V 13, ○——○ A 7. Points obtained from pooled samples from 5 mice.

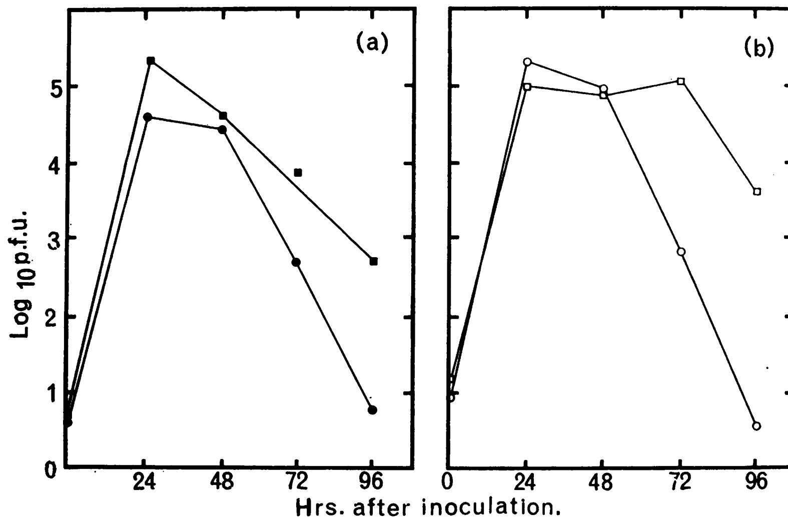

Fig. 3.—Infectivity of mouse spleens (pfu/g.) and that in their contained blood (pfu/0·17 ml.) after inoculation of virulent (V 13) and avirulent (A 7) SFV. (a) V 13, ■——■ spleen, ●——●, blood. (b) A 7, □——□ spleen, ○——○ blood. Points obtained from pooled samples from 5 mice.

infection process 72–96 hr after inoculation (Fig. 3). However before 72 hr there was an indication that splenic suspension of V 13 had infectivities above those equivalent to the contained blood whereas the splenic suspensions of A 7 may have had lower infectivities than those equivalent to their contained blood. Although variation occurred, the results of experiments with the blood and spleens of individual mice (Table) confirmed those with pooled samples. Comparison of the blood contents of virus for A 7 and V 13 at corresponding times showed a tendency for A 7 to be above V 13, significantly so at 48 ($P = 0{\cdot}1$ per cent) and 72 hr ($P = 2$ per cent). The spleen contents of both strains were greater than those of their contained blood late in the infection and early in

TABLE.—*Virus Contents of the Blood and Spleens of Individual Mice at Intervals after Intraperitoneal Injection of Virulent (V 13) and Avirulent (A 7) Strains of SFV*

		Infectivities (log 10 pfu) of			
		A7		V13	
Time after inoculation hr	Mouse number	In blood (0·17 ml.)	In spleen (1 g.)	In blood (0·17 ml.)	In spleen (1 g.)
24	1	6·11	5·66	4·27	4·48
	2	6·53	6·62	5·23	5·97
	3	5·79	5·69	5·27	5·92
	4	4·29	5·21	5·39	5·64
30	1	6·13	5·91	5·51	5·74
	2	5·73	5·11	5·58	5·53
	3	3·57	2·96	4·76	5·51
	4	7·70	5·40	4·73	5·13
36	1	4·77	4·35	4·74	4·78
	2	5·59	5·05	5·67	4·72
	3	3·04	2·82	4·39	3·05
	4	5·29	6·79	4·68	4·60
48	1	5·27	5·05	3·83	4·08
	2	4·96	5·07	3·77	4·88
	3	5·58	4·41	4·91	5·16
	4	6·56	5·98	4·78	4·75
72	1	2·77	4·53	2·98	2·93
	2	3·46	5·20	2·35	3·51
	3	4·65	5·63	2·47	3·67
	4	3·59	5·30	3·36	4·18

infection there was a tendency for the spleen contents of V 13 to be higher and those of A 7 lower than infectivities equivalent to their contained blood.

The virus content of the brain homogenates from mice receiving V 13 became significantly greater than that of the contained blood at approximately 30 hr after inoculation and increased to 10^8–10^9 pfu/g. by the time the mice were dying at 72–96 hr (Fig. 4a). In contrast, brain homogenates from mice receiving A 7 did not exhibit more virus than that of the contained blood until 36–48 hr after inoculation; and at 72–96 hr the virus content, approximately 10^6 pfu/g., was much lower than that of V 13 (Fig. 4*b*).

After inoculation both strains of virus rapidly invaded the peritoneal wall and replicated to high infectivity with A 7 showing levels above those of V 13 at 24–48 hr after inoculation but with the reverse pattern at later times (Fig. 5*a*). High levels of both strains of virus were also found in hind limb muscle, the patterns

of infection being similar to those in peritoneal wall (Fig. 5*b*). Both strains were also present in aorta wall and from 24 hr after inoculation the infectivities of V 13 were higher than those of A 7 (Fig. 5*c*). The ability of both strains to replicate

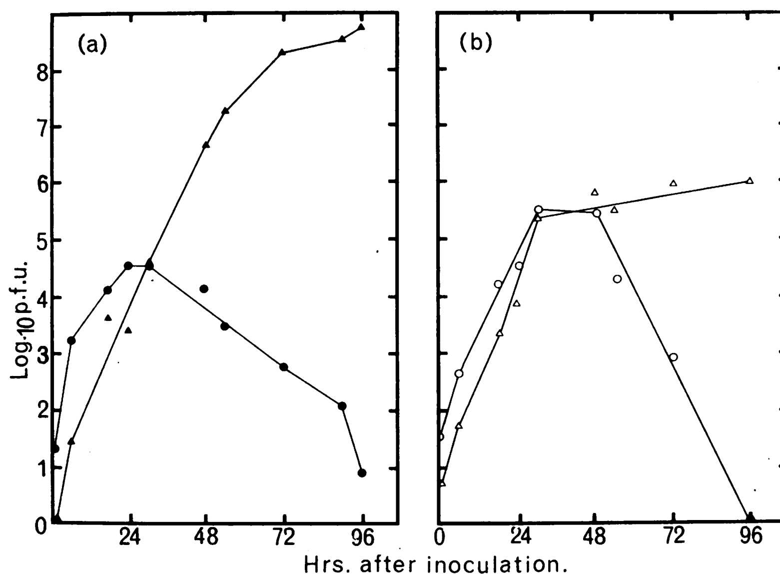

FIG. 4.—Infectivity of mouse brains (pfu/g.) and that in their contained blood (pfu/0·03 ml.) after inoculation of virulent (V 13) and avirulent (A 7) SFV. (a) V 13, ▲——▲ brain, ●——● blood. (b) A 7, △——△ brain, ○——○ blood.

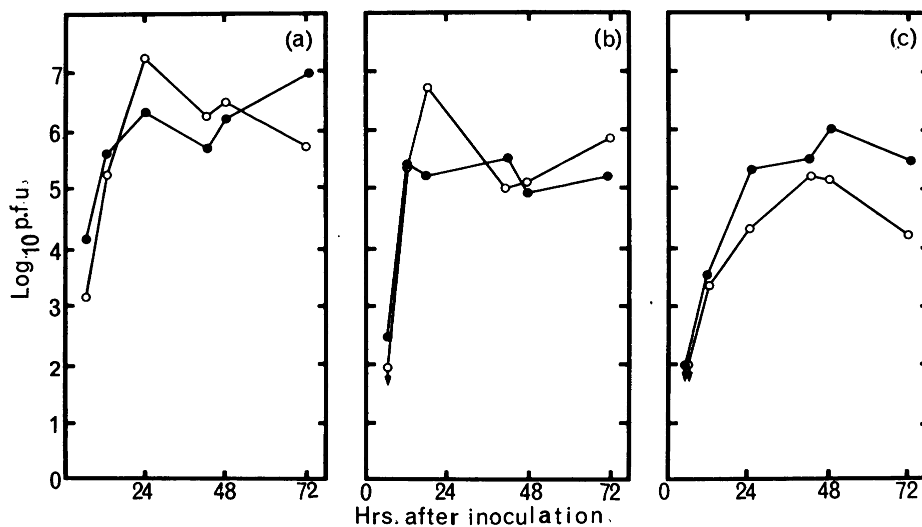

FIG. 5.—Infectivity of mouse peritoneal wall, hind limb muscle and aorta (pfu/g.) after inoculation of virulent (V 13, ●——●) and avirulent (A 7, ○——○) SFV. (a) Peritoneal wall. (b) Hind limb muscle. (c) Aorta.

in aorta wall was confirmed by studies in organ culture. Under the conditions described by Basarab and Smith (1970) for growth of influenza virus in ferret tissue, both strains of SFV infected fragments of mouse aorta and significant quantities (10^3–10^5 pfu per dish) of virus were liberated into the supernatant fluid each day for 2–5 days after inoculation (2×10^3 pfu per dish).

Virus infectivities in the blood and brain after intracerebral inoculation

The virus content of the blood of mice was estimated at intervals after i.c. inoculation of 10^4 pfu of the SFV strains. The blood infectivities were similar to those observed after i.p. inoculation and again the maximum titre of A 7 was greater than that of V 13 (Fig 6*a*). Fig. 6*b* shows the virus contents of the

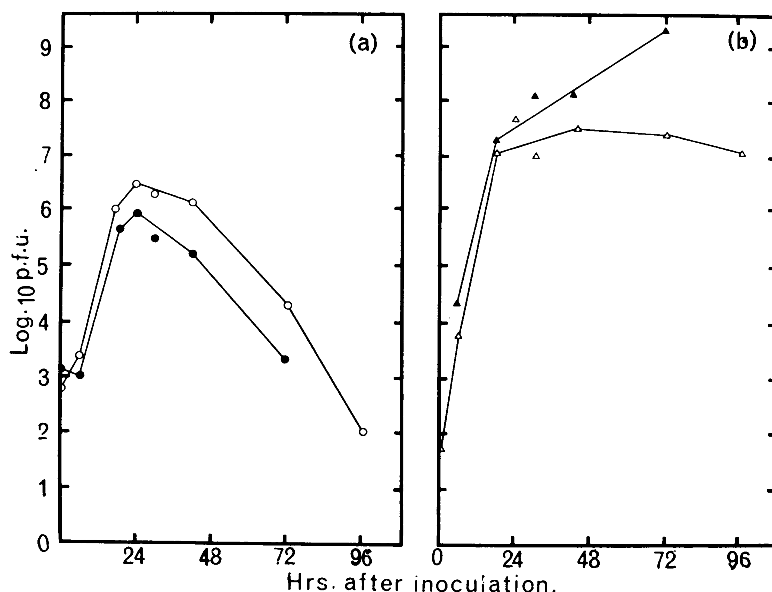

FIG. 6.—Infectivity of mouse blood and brains after i.c. inoculation of virulent (V 13) and avirulent (A 7) SFV. (a) Blood (pfu/ml.) V 13, ●——● A 7, ○——○. (b) Brain (pfu/g.; corrected for virus in the contained blood, pfu/0·03 ml.) V 13 ▲——▲, A 7 △——△.

brains of the mice after correction for the virus content of the contained blood. The replication of V 13 and A 7 appeared the same up to 12 hr after inoculation when the brain content was approximately 10^7 pfu/g.; thereafter V 13 continued to increase to a high titre when the mice died whereas the content of A 7 remained at approximately 10^7 pfu/g. until the third day and then decreased.

Protection of mice against the virulent strain by a prior injection of the avirulent strain

When A 7 (10^4 pfu) was injected i.p. into groups of mice (20) at the same time and 6, 12, 24, and 48 hr before V 13 (10^4 pfu), nil, 3, 10, 12 and 19 mice survived respectively compared with none in a group receiving V 13 (10^4 pfu) alone; observations were made over 10 days and 17 mice survived in a group receiving

A 7 (10^4 pfu) alone. Corresponding figures for survivors after i.c. inoculation of the two strains at the above intervals were 0, 0, 0, 11 and 18.

DISCUSSION

The results reported here support the recent work of Murphy *et al.*, (1970); a generalized growth of SFV occurred mainly in muscle and this provided the reservoir for a viraemia leading to brain invasion. Immediately after i.p. injection of both strains, the peritoneal wall was invaded and prolific virus replication occurred; the amount of virus in the peritoneal wall (estimated at 0·5–1 g. for a 30 day old mouse) was much greater than that in the peritoneal cavity (Figs. 1 and 5) where the virus was not cell-borne but free in the fluid. Similarly the blood was merely a carrier for the virus and practically no virus was found in the cells (see also Mims, 1964). Even after i.c. inoculation, the virus content of the blood indicated that a generalized infection occurred similar to that proceeding after i.p. inoculation.

Bradish *et al.*, (1971) showed that the avirulent and virulent strains were similar in their efficiency of replication from a small inoculum. Now it is clear that the generalized infection produced by the avirulent strain during the first 24–48 hr of infection was as high as that with the virulent strain and possibly higher. This is surprising and contrasts with the more common pattern found in other bacteriological and virological studies including those on arboviruses (Huang and Wong, 1963; Campbell, Buerra and Tobias, 1970). Yet, despite the similar situations regarding generalized infection, V 13 produced significant replication in the vital site—the brain—at 36–48 hr approximately 12 hr before significant replication of A 7 was detected (Fig. 4). Furthermore brain infection by V 13 increased until death but fell for A 7. Thus, the superior ability of the virulent strain to replicate prolifically in the brain having breached the " blood brain barrier " was not due to a superior ability to mount a high viraemia as has been suggested for virus attack on the brain by Johnson and Mims (1968).

In seeking an explanation for the different extent of brain infection achieved by the 2 strains, the possibility that these strains may have a different ability to replicate in brain tissue is unlikely since they behaved similarly in early growth in the brain after i.c. inoculation (Fig. 6*b*). It is possible that the 2 strains have a different ability to replicate in vascular endothelium resulting in different abilities to invade brain tissue. The results in Fig. 5 suggest that, in contrast to the behaviour in peritoneal wall and leg muscle, the virulent strain may replicate faster than the avirulent strain in the muscular aorta wall and this may reflect superiority of the virulent strain to invade vascular endothelium. However, the most likely explanation lies in the relative ability of the strains to evoke or resist body defence mechanisms mobilized within the first 24 hr of infection; that such defences are mobilized very rapidly is indicated by the protection of mice by A 7 infection against an otherwise lethal challenge with V 13 given only 12 hr later (cf. Bradish, Allner, Pusztai, Gould and Smith, 1970; Bradish and Allner, 1971*b*). If A 7 either stimulated more powerful host defence mechanisms or was more prone to these mechanisms than V 13, then its infection would begin to wane, as compared with that of the virulent strain, 24–48 hr after infection, *i.e.* about the time when the crucial growth of virus in the brain is detectable. At 48 hr after inoculation there is clear evidence that A 7 infection is reducing in all

tissues. Furthermore, there is a suggestion from the results in Fig. 3 and the Table that during the first few days of infection spleen tissue may destroy A 7 in its contained blood in contrast to its action on V 13.

Apart from those in the Table, the results reported here were obtained from pooled samples of blood and tissues from groups of 5 young mice injected with a standard high inoculum (10^4 pfu) of virus strains. Variation between individual mice occurred (Table) and the results of pooled samples would reflect mainly the higher infectivities of individual constituents. However the results reported are typical of many experiments with pooled samples all of which produced similar results; and they are supported by the results from individual mice (Table). Furthermore similar conclusions for the patterns of blood, spleen and brain infectivities were obtained from observations on individual mice in parallel experiments involving mice of different ages inoculated by different routes with small and large doses of the 2 strains and with a different method of correction of the infectivities of spleen and brain for virus in their contained blood (Bradish and Allner, 1971*a*).

We thank Mr. S. Peto for statistical analyses of results in the Table and Dr. J. Smillie for help with some experiments. We are indebted to Dr. C. J. Bradish for supply of materials and many fruitful discussions.

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