THE INVASION OF THE BRAIN BY YELLOW FEVER VIRUS PRESENT IN THE BLOOD OF MICE

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ADULT mice are highly susceptible to intracerebral but relatively insusceptible to peripheral inoculation with most arthropod-borne viruses. Infant mice are highly susceptible to either type of inoculation, and the change-over to the adult response usually occurs during the second and third weeks of life (Bugher, 1941 ; Lennette and Koprowski, 1944). Attempts to define the " barrier " which develops in adult animals have not been very successful. During a study of the ability of peripherally inoculated yellow fever virus to invade the central nervous system of mice, results were obtained which help define this barrier, and in addition suggest the route by which the central nervous system is invaded.

MATERIALS AND METHODS

Standard diluent.—The standard diluent was 0·75 per cent bovine plasma albumin in saline buffered at pH 7·4.

Virus.—The Asibi strain of yellow fever virus was used in the form of serum from infected rhesus monkeys. Titres were found to be steady for at least a week when serum was stored in the frozen state at — 20°.

The French Neurotropic strain of yellow fever virus was in its approximately six-hundredth mouse passage, and virus was obtained as centrifuged mouse brain suspension. Suspensions were made in standard diluent and were used when fresh.

Mice.—The mice used were of Swiss stock, their ancestors originating from Carworth Farms, New York. Adults were inoculated when from 30–35 days old.

Infectivity titrations.—Serial tenfold dilutions of infective material were made in standard diluent, and each dilution inoculated (0·03 ml.) intracerebrally into 5 or 6 adult mice. Mice were observed for 12 days (French Neurotropic) or 16 days (Asibi) and end-points then calculated from deaths by the method of Reed and Muench (1938). Deaths which occurred on the first day (French Neurotropic) or during the first 4 days (Asibi) were regarded as non-specific and were not included in calculations. Death times were recorded from daily or twice daily observations.

In preliminary experiments, four different stock Asibi virus sera were titrated subcutaneously and intracerebrally in 3-day-old mice, and two of them intracerebrally in adult mice as well. Non-specific deaths were more common in infant mice, and two litters were therefore inoculated with each dilution. In Table I, where all titres are corrected to $\log_{10}$ LD_{50}/0·03 ml., it can be seen that infant mice are rather more susceptible by the intracerebral than by the subcutaneous route, and even by the subcutaneous route they are about ten times as susceptible as are adult mice by the intracerebral route. Nevertheless, adult mice were used in titrations, because they were more readily obtainable and only half as many were required.

Organs were made up as 10 per cent suspensions in standard diluent, and the suspension considered as a 10^{-1} dilution in titrations, so that titres are expressed in LD_{50} per 0·03 ml. of solid tissue. Gall-bladders were discarded when adult mouse livers were removed, but this was not possible with infant mouse livers. Antibiotics, when used, were added so that each ml. of inoculum contained 1000 units of soluble penicillin and 1 mg. streptomycin.

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TABLE I.—*Titrations of Asibi Virus in Adult and Infant Mice*

	Titration method		
		3-day mouse	
	Adult, intracerebral	Subcutaneous	Intracerebral
Stock serum 1	5·0	5·7	6·3
,, ,, 2	6·3	7·6	—
,, ,, 3	—	2·3	3·2
,, ,, 4	—	6·3	6·9

EXPERIMENTAL

The Growth of Yellow Fever Virus in Infant Mice

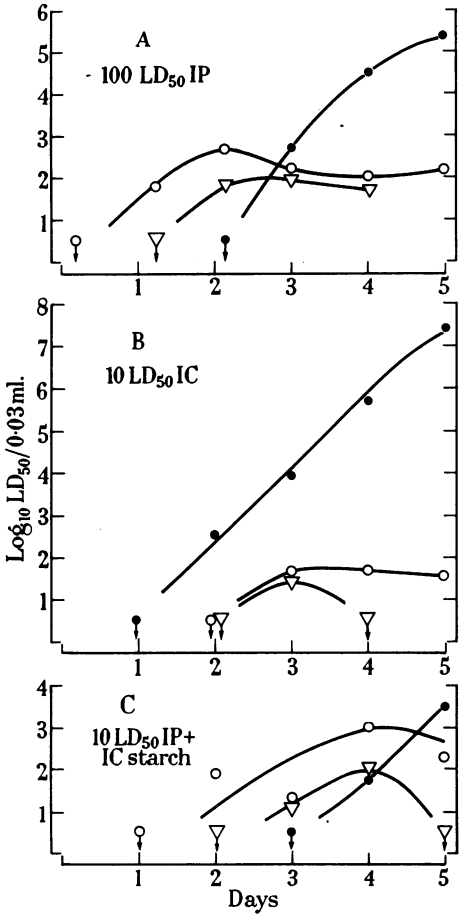
It was shown by Theiler (1930) that infant mice, unlike adults, are highly susceptible to peripherally injected yellow fever virus. When infants were inoculated intraperitoneally, virus first appeared in the brain by 48 hr. and pooled suspensions of liver, spleen and kidney were found to contain virus at 24 hr. but not later than this. Theiler thought that the virus present at 24 hr. might have been the residuum of the originally inoculated virus which was still present on the peritoneal surface of these organs. The experiments described below were done to see whether or not there was evidence for the visceral multiplication of virus in infant mice.

The effect of the route of inoculation.—Many litters of one-day-old mice were inoculated intraperitoneally with 100 LD_{50} of Asibi virus contained in 0·02 ml. of standard diluent. At intervals 5 infant mice, each from a different litter, were killed with ether and the bloods, livers and brains separately pooled and titrated. In the growth curves obtained (Figure A) it can be seen that the liver titre rose 1–2 days earlier than the brain titre, and then fell slightly while the brain titre continued to rise. Blood titres followed liver titres fairly closely.

In another experiment 10 LD_{50} of Asibi virus were injected intracerebrally (0·02 ml.) into one-day-old mice, and growth curves again obtained in the same way. Mice which were not sacrificed were observed, and the times of sickness and death recorded. It can be seen (Figure B) that blood and liver titres behaved much as in the previous experiment, although with the smaller dose inoculated a longer latent period preceded the rise in titres. Virus now appeared in the brain about a day earlier than in the liver. The median death time in 15 unsacrificed mice was 8·0 days.

Finally, about 10 LD_{50} of Asibi virus were injected (0·02 ml.) intraperitoneally into 14 litters of one-day-old mice. Immediately afterwards 0·02 ml. of 2 per cent starch solution was given intracerebrally to 11 of these litters, from which mice were taken at intervals and blood, liver and brain growth curves obtained in the usual way. The growth curves (Figure C) were similar to those obtained with intraperitoneal virus alone (Figure A), the longer latent period being attributable to the smaller dose inoculated. The intracerebral starch, therefore, had not affected the pattern of growth. Sickness and death was recorded in 21 unsacrificed mice as well as in the 3 litters which did not receive intracerebral starch. The median death time was 9 days in each group of mice, a day later than in the preceding experiment in which the same amount of virus was given intracerebrally. This difference in death time was confirmed when mice were inoculated either intraperitoneally or intracerebrally with an identical inoculum.

Four litters of one-day-old mice were given $10^{7\cdot0}$ LD_{50} intraperitoneally and their median death time (7 days) was a day later than for another 4 litters which had been given exactly the same dose intracerebrally.



FIGURE—Growth curves for Asibi virus in the liver (circles), blood (triangles) and brain (dots) of infant mice. Inocula and inoculation routes as shown. At each time interval five mice were sacrificed and titrations made on pooled material.

These experiments show that when Asibi virus is administered intraperitoneally to infant mice, 1–2 days' growth in the viscera precedes growth in the brain, even when the brain is traumatized by an injection of starch. When virus is deposited directly in the brain it begins to grow there before there has been visceral growth and death occurs about one day earlier. There is the same difference in death time even when large inocula are given. The greater part of an intracerebral inoculum spills over immediately into the blood stream (Cairns, 1950) and accordingly visceral growth occurred in the usual way after the intracerebral inoculation.

The route of invasion of the central nervous system.—An experiment was done in an attempt to find out which part of the central nervous system was first invaded by intraperitoneally injected virus. Many litters of 3-day-old mice were inoculated intraperitoneally with 100 LD_{50} of Asibi virus. At intervals 5 mice from different litters were killed with ether and different parts of their nervous systems removed, pooled and titrated in the usual way. The brains were divided into anterior and posterior parts so that the posterior part comprised the cerebellum, pons and medulla, and the spinal cords were taken from the thoraco-lumbar region. It was found (Table II) that although there was no detectable

TABLE II.—*The Growth of Asibi Virus in the Brains of Baby Mice after Intraperitoneal Inoculation*

Time (hr.)	Titres of the parts tested		
	Anterior brain	Posterior brain	Spinal cord
24	$<1{\cdot}0$	$<1{\cdot}0$	$<1{\cdot}0$
53	$<1{\cdot}0$	$<1{\cdot}0$	$<1{\cdot}0$
72	$<1{\cdot}0$	$<1{\cdot}0$	$<1{\cdot}0$
78	1·6	1·0	1·5
96	3·0	1·6	1·0
120	4·0	3·5	3·8

virus in the 10 per cent suspensions at 72 hr., each of them contained small amounts of virus by 78 hr. Thenceforth the titres of the parts did not differ significantly, except at 4 days, and the importance of this particular difference is uncertain. It is concluded that Asibi virus injected intraperitoneally in infant mice either reaches each main part of the central nervous system at the same time, or alternatively spreads rather rapidly throughout the central nervous system from a single site of entry.

The Growth of Yellow Fever Virus in Adult Mice

Response to small inocula.—In the first experiment adult mice were inoculated subcutaneously (0·03 ml.) with 100 LD_{50} of Asibi virus. Five hours later, and then each day for 5 days, 5 or 6 mice were killed with ether and the pooled sera, livers, and brains each tested for virus by inoculating 3-day-old mice subcutaneously (0·03 ml.). Virus was at no time detected either in undiluted sera or in 10 per cent liver suspensions and brain suspensions.

In the next experiment, a similar dose was injected intracerebrally into adult mice, and at the same time intervals sera and livers were again tested for virus by inoculating infant mice ; on this occasion brains were titrated intracerebrally in adult mice. Detectable virus appeared in the brain by 24 hr. and the titre then gradually increased, reaching $10^{6{\cdot}2}$ LD_{50}/0·03 ml. brain by the fifth day. No virus was detected in any of the 10 per cent liver suspensions but there was evidence that small amounts were present in serum on the second and fifth days. Some of the infant mice inoculated with these undiluted sera sickened or died after from 10 to 12 days and their pooled, seitz-filtered brain suspensions killed adult mice within 8–12 days on intracerebral inoculation.

In two more experiments where similar amounts of virus were injected intravenously and intraperitoneally into adult mice, and liver and serum pools tested once more in infant mice, virus was not isolated from liver pools and there was no evidence of viraemia.

These experiments show that when a small dose of Asibi virus is administered either intravenously, intraperitoneally, or subcutaneously into adult mice, virus is not at any time detectable in the liver or serum. There was probably a very low grade viraemia following intracerebral inoculation but this might have been due to a leakage of virus from the brain. Thus, while virus multiplies in the brain following intracerebral inoculation there is little or no visceral multiplication, whatever the route of inoculation.

Attempt to adapt virus to grow viscerally in adult mice.—Because Asibi virus grew viscerally in infant but not in adult mice it was thought possible that a series of passages in the viscera of infant mice might increase the ability of virus to grow viscerally in adult mice. About $10^{4\cdot0}$ LD_{50} of Asibi virus were injected intraperitoneally into 3 litters of one-day-old mice and 3 days later, when virus is known to be present in liver and blood (Figure A and C), 5 mice were sacrificed and the pooled livers suspended in standard diluent and centrifuged. After the addition of antibiotics the suspension was titrated intracerebrally in adult mice and injected intraperitoneally into another series of one-day-old mice. Livers from these mice in turn were dealt with in the same way so that a viscerotropic passage series was initiated. Later in the series mice were inoculated subcutaneously as well as intraperitoneally, and passages were made at 4-day intervals ; on five occasions livers contained very little virus at the time of sacrifice and brains from mice sick at 9 days were then passaged. After 32 passages, the peak liver titres attained in infant mice had not altered, and remained less than $10^{3\cdot0}$ $LD_{50}/0{\cdot}03$ ml. liver. Nor had there been any change in the response of adult mice to small intraperitoneal inoculations, for they were still insusceptible and still failed to develop viraemia on the third day. The tests for viraemia were made in two separate experiments by making a pool of serum from 3 mice and injecting it undiluted into adult mice by the intracerebral route.

There is therefore no evidence that the visceral passage of Asibi virus in infant mice increases its ability to grow viscerally in adult mice.

Response to large inocula.—It was found that adult mice were susceptible to the peripheral administration of Asibi virus if the dose was large enough. The results of many tests showed that a large proportion of adult mice died when inoculated intraperitoneally or intravenously with more than about $10^{6\cdot0}$ LD_{50} of Asibi virus. It was found that about $10^{5\cdot0}$ LD_{50} were lethal if the brain was pierced by a needle at the time of the intraperitoneal inoculation and there was a similar, although less well marked effect, when the spinal cord was pierced in the same way.

Adult mice are more susceptible to the intraperitoneal inoculation of the French Neurotropic strain of yellow fever virus if the brain is damaged at the same time (Sawyer and Lloyd, 1931). It was shown by Goodner and Smithburn (1951) that this occurred even when an intracerebral injection of 2 per cent starch solution was given 3 hr. before or $\frac{1}{2}$ hr. after the virus inoculum. If brain trauma acts by directly exposing central nervous tissue to circulating virus it is not easy to understand why tissues remain exposed for at least 3 hr. It seemed possible, in the light of this finding, that the trauma acts indirectly by locally increasing the permeability of the blood-brain barrier. A series of experiments was therefore done to test this possibility. Randomized groups of 15–20 adult mice were inoculated intraperitoneally with large doses of Asibi virus and at the same time the brains of one group were pierced with a needle. A control group

of mice received nothing except the virus inoculum and other groups were given treatments in attempts to produce local dilatation of the blood vessels in the central nervous system (Trueta and Hodes, 1954). These treatments were: an intramuscular injection of a sterile emulsion of oil of turpentine, the production of a skin-muscle burn in the scalp or on the leg, and the production of fatigued limbs by inducing mice to swim for 40 min. by immersing them in lukewarm water. The results showed that although mice were more likely to die of yellow fever if the brain was pierced with a needle at the time of the virus injection, none of the other treatments had this effect. A similar experiment was done using the French Neurotropic strain, and the results were the same. This strain of virus, like the Asibi strain, was found to invade the undamaged brain when large enough doses were injected intraperitoneally.

Both these strains of virus, therefore, tend to invade the brain of adult mice when large intraperitoneal or intravenous doses are given. This tendency is increased when the brain or spinal cord is directly damaged, but other treatments which might indirectly alter the permeability of the blood-brain barrier are ineffective.

In investigating the ability of peripherally injected virus to invade the brain of adult mice it seemed important to know how long virus stays in the blood. An experiment was therefore done in which $10^{6 \cdot 7}$ LD_{50} of Asibi virus were injected intravenously into 6 adult mice, and at intervals these mice were bled from their tails and the pooled blood titrated. At 1 hr. and at 3 hr., mice were anaesthetized by the intraperitoneal injection of a barbitone-chloral hydrate mixture, then killed by exsanguination from the heart, and thorough nasopharyngeal washings obtained in standard diluent. These washings were centrifuged and after the addition of antibiotics injected intracerebrally into adult mice. The results (Table III) show that nearly all virus had disappeared from the blood within 10

TABLE III.—*The Disappearance of Intravenously Injected Asibi Virus from the Blood of Adult Mice*

Time after inoculation	Blood titre	Fate of mice inoculated with nasopharyngeal washings
0	4·7 (calculated)	—
10 min.	2·9	—
1 hr.	1·0	ssssss ssssss
3 ,,	—	ssssss
8 ,,	<1·0	—

Each s indicates survival of an inoculated mouse.

min., and none was detectable in tenfold diluted blood of the 3 remaining mice at 8 hr. No mice inoculated with nasopharyngeal washings died, and it is concluded that little or no virus was present on the nasal mucosa at the times tested. Virus was injected intravenously in this experiment and disappeared from the blood so rapidly that there would probably be little chance of detectable viraemia being established after intraperitoneal inoculation where there was a gradual release of virus into the blood. Nevertheless, virus may enter the brain after large inocula, whether they are given intraperitoneally or intravenously and it may be that the extent and duration of viraemia is not of great importance in this connection.

In another experiment in which approximately $10^{7.0}$ LD_{50} of virus were injected intraperitoneally into 6 adult mice, and daily pooled tail blood samples diluted tenfold and tested in adult mice, viraemia was not detected. Two mice were sacrificed on the fourth day, and their livers were pooled and the titre found to be $10^{1.1}$. Two of the remaining 4 mice died, on days 10 and 11. On another occasion 2 mice were given $10^{5.0}$ LD_{50} intraperitoneally and their pooled liver titre on the fourth day was $10^{2.0}$. It seems possible, therefore, that the liver plays a part in removing virus from the blood.

Comparison of the ability of neurotropic and pantropic virus strains to infect adult mice by the nasal route.—Several viruses have been shown to invade the central nervous system from the nasal mucosa *viâ* the olfactory tracts. Sometimes these results have been difficult to interpret because neurotropically adapted strains of virus were used. The Asibi and French Neurotropic strains were titrated intracerebrally and intranasally in adult mice and the results (Table IV)

TABLE IV.—*Comparative Titrations of French Neurotropic and Asibi Strains in Adult Mice by Intracerebral and Intranasal Routes*

Virus	Titres	
	Intracerebral	Intranasal
Asibi	5·0	<0*
French Neurotropic	7·0	3·1

* No mice receiving undiluted virus died.

show that, whereas for French Neurotropic $10^{4.0}$ intracerebral LD_{50} are equivalent to an intranasal LD_{50}, more than $10^{5.0}$ intracerebral LD_{50} of Asibi are required. The neurotropically adapted strain therefore infects more readily than the pantropic strain when administered intranasally.

DISCUSSION

It has been shown that when the Asibi strain of yellow fever virus was injected intraperitoneally into infant mice there was a period of 1–2 days' visceral growth as shown by a rise in liver and blood titres, before virus was detectable in the brain. When virus was injected directly into the brain it multiplied there without this period of delay, and death occurred approximately one day earlier. It is suggested that the period of visceral growth is necessary before peripherally introduced virus can reach the central nervous system. Adult mice would then be insusceptible to peripherally introduced virus because, as has been shown, this visceral growth probably does not occur. The fact that they are susceptible to exceedingly large peripheral inoculations will be discussed later. Thus, the " barrier " which develops in adult mice would involve the loss of cells which support the visceral multiplication of virus. It is noteworthy that Semliki Forest virus, one of the few arthropod-borne viruses which readily kill adult mice on peripheral inoculation (Smithburn and Haddow, 1944), has been shown to undergo a period of visceral growth before it appears in the brain. When $10^{3.0}$ LD_{50} of this virus were injected intraperitoneally in adult mice a rise in blood titre occurred 1–2 days before a rise in brain titre (M. C. Williams, personal communication).

In the experiments where adult mice were given small extraneural inocula there was no evidence for the multiplication of virus, in spite of the fact that the tests were made particularly sensitive by the use of infant mice. Injected virus, however, was shown to disappear rapidly from the blood of adult mice, and thus the rate of entry of virus into the blood must also be rapid if viraemia is to be detected. It is therefore conceivable that virus was released into the blood in these experiments but was removed too rapidly for virus to be detected. If there had been visceral multiplication it is unlikely that it took place in the liver, for virus was never detected in this organ. Moreover, even if virus had been released into the blood from other organs or tissues, the liver might have been expected to contain virus which it had taken up from the blood ; it was shown in another experiment that virus was still present in liver suspensions 4 days after a large intraperitoneal inoculation. It is therefore thought improbable that visceral multiplication occurs in adult mice.

Why should 1–2 days' visceral multiplication be necessary before Asibi virus can reach the central nervous system of infant mice? Possibly in infants the viraemia lasts long enough for virus to be excreted on the nasal mucosa, and the brain is then invaded by way of the olfactory tracts but, for the reasons given below, it is thought unlikely that the nasal mucosa is important. If, however, virus grew in the endothelium of capillaries in the central nervous system and was then released both back into the blood and into the adjacent nervous tissue, it would have reached nervous tissue as a direct consequence of visceral growth. It may be that in infant, but not in adult mice, these capillary endothelial cells support virus multiplication. In the growth curve experiments with infant mice, liver titres were consistently slightly higher than blood titres, so that multiplication may therefore occur in the liver but it would not necessarily occur exclusively there.

Two of the routes by which viruses present in the blood might reach the central nervous system were clearly envisaged many years ago by Hurst (1936) and by King (1939). Either virus crosses the blood-brain barrier at one or more sites, or it reaches the brain by way of the olfactory tracts after being excreted on to the nasal mucosa. Clearly the question is of great importance in understanding the occurrence of encephalomyelitis in man following infection with arthropod-borne viruses or poliomyelitis virus, but experimental evidence has sometimes been difficult to interpret. For one thing, neurotropically adapted rather than recently isolated strains have often been used and, as has been shown above for the French Neurotropic strain of yellow fever, such strains may be unusual in their ability to infect by the nasal route. The fact that after the peripheral inoculation of infant mice, Asibi virus appeared in each main part of the central nervous system at about the same time, suggests that the nasal mucosa is not important in this instance. Adult mice are so insusceptible to intranasal inoculation that, even if large amounts of virus were excreted on to the nasal mucosa, it is unlikely that it would then reach the brain.

If, as is suggested, virus reaches the central nervous system of infant mice by " growing through " blood vessels (Hurst, 1936), two other experimental findings become easier to understand. First, a widespread release of virus from blood vessels into the substance of the central nervous system would initiate growth far more effectively than an intracerebral inoculation which merely flooded the needle track and the cerebrospinal fluid with virus (Mims, unpublished

observations). It might therefore be expected that virus would reach all available cells and kill the mouse more rapidly, when it entered central nervous tissue at many points from blood vessels. There is evidence that this is, in fact, so, because although after an intraperitoneal inoculum virus appeared in the brain 2–2½ days later than after an intracerebral inoculum of the same size (Figure B and C), the death time was delayed only by one day. Second, it was shown that intracerebral starch did not significantly alter the period of visceral growth which preceded the growth of Asibi virus in the brain of infant mice. This would be expected if, as a result of visceral multiplication there was a widespread release of virus into the brain which made the very small local entry of virus which might have taken place at the site of the intracerebral starch injection unimportant by comparison.

It is not easy to explain why adult mice are susceptible to large doses of Asibi virus by peripheral routes of inoculation. It was shown that even after intravenous inoculation virus disappears very rapidly from the blood, and there was no detectable virus on the nasal mucosa at the times tested. It is possible that when large doses are given there is enough virus present in the blood for a long enough period for it to enter the central nervous system directly, as a result of the changes in capillary permeability which may be assumed to occur in normal adult mice. In passing the blood-brain barrier in this way virus would " leak " through mechanically, in contrast to the " growth through " which, it is suggested, occurs in infant mice. Since it is possible that different viruses use different routes or a given virus more than one route, these experimental findings should, strictly speaking, be applied only to the virus-host system used in the experiments.

SUMMARY

It has been shown that when small doses of the Asibi strain of yellow fever virus are administered intraperitoneally to infant mice, a period of 1–2 days' visceral multiplication precedes the appearance of virus in the brain. When virus is given intracerebrally, it appears in the brain about a day earlier than in the viscera and death now occurs a day earlier. It is suggested that virus reaches the brain from the periphery as a result of the preceding visceral multiplication, possibly by multiplying in the endothelium of capillary blood vessels in the central nervous system. There is no visceral multiplication in adult mice and it is to this that their insusceptibility is attributed.

When virus is given intraperitoneally to infant mice, it appears in each main part of the central nervous system at about the same time. This is taken as evidence that virus reaches the central nervous system directly from blood vessels.

Adult mice are susceptible to the peripheral inoculation of very large doses of Asibi virus. Smaller doses are sufficient if the central nervous system is traumatized at the time of the inoculation, but treatments designed to alter capillary permeability in the central nervous system do not have this effect.

Intravenously injected virus is removed very rapidly from the blood of adult mice.

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