FACTORS INFLUENCING THE PERSISTENCE OF CHORIOMENINGITIS VIRUS IN THE BLOOD OF MICE AFTER CLINICAL RECOVERY

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In previous publications (1, 2) it was reported that the virus of lymphocytic choriomeningitis persisted in the bodies of some infected mice for several weeks or months after clinical recovery, while it could not be demonstrated in others. Since the carrier problem is of considerable importance in the epidemiology of certain virus diseases and also has a bearing on the mechanism of the immunity to viruses, the factors that influence the duration of the infection in mice were investigated.

The presence or absence of demonstrable virus in the blood was chosen as a basis of comparison, since previous tests have shown that the virus can almost always be detected in the blood of recovered mice if it is present in appreciable amounts in their tissues. This is chiefly due to the absence or exceedingly low concentration of antibodies in the serum of such animals. There are exceptional cases, however, in which the blood is not infectious but virus is excreted in the urine. We have observed two such instances, mice which became infected naturally when very young and were tested for virus in the blood and urine at the age of 4 or 6 months. The kidneys tested in one animal contained virus, which probably accounted for the virulence of the urine. In spite of such exceptions it is believed that the examination of the blood for virus is adequate for comparative purposes.

Methods and Materials

Most of the technical procedures used have already been described, but some of them have since been modified and will therefore be described again.

Tests for Virus in Blood.—The blood to be tested was obtained by heart puncture under deep ether anesthesia. About 10 per cent of the mice were lost through injury, but the others did not seem to be harmed even when the bleedings were repeated at short intervals. In tests with undiluted blood from mature mice, 0.1 cc. heart blood was withdrawn with a 0.25 cc. syringe and a 27 gage needle, and 0.05 cc. was inoculated immediately into the brain of an anesthetized mouse. Mice 5 to 6 weeks of age were used as test animals. They are about equally as susceptible as guinea pigs to blood from carrier mice. The mice that failed to show symptoms after the blood inoculation were tested for immunity by intracerebral injection with highly virulent mouse passage virus given 2 weeks after inoculation, an equal number of normal mice being used as controls. A small portion of the mice so tested proved completely immune, and such animals were considered to have become infected by the blood.

For titration 0.2 cc. heart blood was withdrawn and mixed immediately with 1.8 cc. heparinized saline. Further decimal dilutions were likewise made in heparinized saline. The heparin has no inactivating effect upon the virus and is harmless to mice. The highest decimal dilution causing characteristic symptoms on intracerebral injection was considered as the titration end-point.

Tests for Virus in the Tissues, Nasal Secretions, and Urine.—The carrier mice whose viscera were tested were bled (1.2 to 1.5 cc.), the blood being collected in a test tube containing a trace of heparin. Of the organs removed immediately after death a 10 per cent suspension in saline was made, which was considered a 10^{-1} dilution. The decimal dilutions in saline indicated in Table III were kept in an ice bath during the short period of time between preparation and injection, and 0.05 cc. of each dilution was inoculated intracerebrally into a mouse. As with the blood, which was titrated simultaneously, the highest decimal dilution producing typical symptoms was considered as the titration end-point.

Nasal washings were obtained according to the method already described (1), that is, simply by immersing the nostrils of a mouse 3 times for about 20 seconds in 1 cc. saline contained in a slanted Petri dish. The animal is thus forced to inhale some fluid repeatedly and to blow it out into the remaining fluid, which gradually becomes cloudy. Severe dyspnea usually follows each of the three inhalations but the mice recover rapidly. Some care is necessary, however, to prevent the animals from drowning. The undiluted washing fluid was injected into the hind pads of guinea pigs in amounts of 0.7 cc., and the tenfold dilutions of it in amounts of 1 cc.

Urine was collected in a Petri dish by exerting slight pressure on the rear part of the abdomen in the region of the urinary bladder, and inoculated in the same way as the nasal washings. The dosage of undiluted urine varied with the amount obtained.

Experimental Mice.—The normal white mice used came from the virus-free mouse colony mentioned before (2), which was built up on the progeny of some uninfected mice obtained from the colony in which choriomeningitis was dis-

covered in 1934. This new stock has been kept under close observation, and has been free from the disease since 1935.

Further essential technical details will be given in the text.

The Influence of the Mode of Natural Infection on the Persistence of Virus in the Blood

Choriomeningitis in mice, as we have observed it (2), is essentially a disease of young animals, which become infected either *in utero* or by contact shortly after birth. Mice infected in either way may carry the virus in the blood for considerable periods of time after recovery. According to observations made in 1935 the number of carriers in the infected stock decreased with increasing age (2). In tests made late in 1937, however, all the mice tested, young and old alike, were carriers.

In the following experiments an attempt was made to determine whether the mode of natural infection had any influence on the duration of the infection in the animals.

Seven pregnant mice 4 to 5 months of age, looking healthy and vigorous, and known to carry virus in the blood, were removed from the infected stock, each animal being placed in a small breeding cage. 2 young ones were taken from each litter immediately after birth and their brains suspended, each in 2 cc. saline. The suspensions were tested for virus by intracerebral inoculation of 2 mice with 0.05×10^{-3} cc. Virus was detected in every case, and the litters were therefore assumed to have become infected *in utero*. It was known from two previous experiments that in litters infected in this manner all the young are infected when born.

The young mice infected *in utero* showed no definite signs of illness except a slightly decreased growth rate in comparison with mice of the same age born from normal mothers. In no instance did these show the severe disease frequently seen in mice infected *in utero* in 1935 (2).

When the 7 litters just mentioned (marked A to G in Text-fig. 1) were 17 to 23 days of age, a new-born normal litter together with the mother was exposed to each of them. The exposed litters marked H to N are recorded in Text-fig. 2. Some of the young of these litters were devoured by the other mice during the first days of life, but enough animals remained to continue the experiment. To determine whether the exposed young mice had become infected by contact with those infected *in utero*, 1 young of each litter was killed on the 10th day after exposure and a suspension of the brain in 2 cc. saline was tested for virus by intracerebral mouse injection. The brains of 5 of the 7 young mice tested contained

Date of bleeding	Litter A Born 11/19/36				Litter B 12/17/36					Litter C 12/23/36			Litter D 12/29/36				Litter E 12/31/36		Litter F 1/1/37				Litter G 1/9/37				
	1 ♂ +	2 ♂ +	3 ♀ +	4 ♀ +	1 ♀ +	2 ♀ +	3 ♀ +	4 ♀ +	5 ♀ +	1 ♀ +	2 ♀ +	3 ♀ +	1 ♂ +	2 ♀ +	3 ♀ +	4 ♀ +	1 ♀ +	2 ♀ +	1 ♂ +	2 ♂ +	3 ♂ +	4 ♂ +	1 ♂ +	2 ♂ +	3 ♀ +	4 ♀ +	5 ♀ +
At age of 3-4 weeks	●	●	●	●	●	●	●	●	●	●	●	ɸ	ɸ	●	●	●	●	●	●	●	●	ɸ	●	●	●	●	●
4/20/37	●	●	●	ɸ	●	●	●	●	-●-	●	●			●	●	●	●	●	●	●	ɸ		ɸ	●	●	●	●
10/11/37	●	●	●		●	●	●	-●-		●	●			●	●	●	●	●	●	●				●	●	●	●
11/4/37	● * 10^{-3}				● 10^{-3}	● 10^{-3}																					
11/18/37		● ** $10^{-3}+$	● $10^{-3}+$				● $10^{-3}+$			● $10^{-3}+$	● $10^{-3}+$			● $10^{-3}+$	● $10^{-3}+$	● $10^{-3}+$			● $10^{-3}+$	ɸ $10^{-3}+$				● $10^{-3}+$	● $10^{-3}+$	● $10^{-3}+$	● $10^{-3}+$
1/17/38	● $10^{-4}+$	● $10^{-4}+$	● $10^{-4}+$		● $10^{-4}+$	● $10^{-4}+$	● $10^{-4}+$			-●- $10^{-4}+$	● 10^{-3}			● $10^{-4}+$	● $10^{-4}+$	● 10^{-3}	● $10^{-4}+$	● $10^{-4}+$	● $10^{-4}+$					● $10^{-4}+$	● $10^{-4}+$	● $10^{-4}+$	● 10^{-3}
1/27/38			● 10^{-5} *i*				● 10^{-4} *i*									● 10^{-3} *i*	● 10^{-5}	● 10^{-3} *i*						● 10^{-4}	● 10^{-4} *i*	● 10^{-4} *i*	

TEXT-FIG. 1. Persistence of virus in the blood of mice infected *in utero*.

Date of bleeding	Litter H Born & exposed 12/11/36				Litter I 1/9/37 2D					Litter J 1/9/37 1D			Litter K 1/20/37 1D				Litter L 1/21/37 3D		Litter M 1/21/37 3D				Litter N 1/31/37				
	1 ♂ +	2 ♂ +	3 ♀ +	4 ♀ +	1 ♀ +++	2 ♀ +++	3 ♀ +++	4 ♀ +++	5 ♀ +++	1 ♀ +++	2 ♀ +++	3 ♀ +++	1 ♂ +++	2 ♀ +++	3 ♀ +++	4 ♀ +++	1 ♂ +++	2 ♀ +	1 ♂ +++	2 ♂ +++	3 ♂ +++	4 ♂ +++	1 ♂ ++	2 ♂ ++	3 ♀ +	4 ♀ +	5 ♀ +
At age of 3-4 weeks	●	●	●	●	●	●	●	●	ɸ	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
4/20/37	ɸ	●	●	○	●	●	○	○		●	●	-●-	●	●	●	ɸ	●	ɸ	●	●	○	○	●	●	●	●	●
10/11/37		ɸ	●	○ *i*	ɸ	-●-	○ *i*	○ *i*		●	●		●	●	○		-●-		●	Φ	○ *i*	● *i*	●	●	●	ɸ	○
11/18/37			● ** $10^{-3}+$							● $10^{-3}+$	● $10^{-3}+$		-●- $10^{-3}+$	○	○ *i*				● $10^{-3}+$				-●- $10^{-3}+$	● $10^{-3}+$	● $10^{-3}+$		● 10^{0}
12/28/37														● 10^{0}													
1/17/38			● * 10^{-3}							-●- 10^{-3}	● $10^{-4}+$								● $10^{-4}+$					● 10^{-3}	● 10^{-3}		
1/27/38											● 10^{-4} *i*														● 10^{-3}		

● = Heart blood infectious.
○ = Heart blood not infectious.
ɸ or Φ = Mouse died from injury through heart puncture.
-●- = Mouse died naturally some time after bleeding.
* = Highest decimal dilution producing characteristic symptoms on intracerebral injection in a mouse.
** = Titration endpoint not reached at this dilution.
i = Completely immune to intracerebral injection with virus.

TEXT-FIG. 2. Persistence of virus in the blood of mice infected by contact shortly after birth.

virus. Those from litters K and M gave a negative result, but these litters nevertheless became infected as shown by the results of later blood tests.

The severity of the resulting disease indicated by the number of + signs in the text-figures varied with different litters. The mice of litters I, J, K, L, and M contracted a severe disease and several of them died, while those of litters H and N showed a less severe reaction, a slightly decreased rate of growth (marked by one +) being the only sign of disease in litter H. All of the sick mice had completely recovered by the end of the 5th week and later looked like normal, uninfected animals. At the age of about 10 months several mice of litters A to N began to show signs of old age, such as lack of liveliness, adiposis, and ruffling of the fur. On the whole, the animals appeared to age sooner than uninfected mice.

The mice of the corresponding litters (A and H, B and I, and so on) were kept together until the contact litters were 3 to 4 weeks old, when the males and females of each litter were separated. The mice of litters A to G, exceeding in numbers those of the corresponding contact litters, were removed and tested for immunity by intracerebral inoculation with virus. They all proved completely resistant and were then discarded.

The blood of the mice of litters A to N was tested for virus for the first time at the age of 3 to 4 weeks, when all the animals carried virus in the circulation. The results of the later tests for virus and immunity are recorded in the text-figures and need not be reviewed in detail. The mice tested for immunity were completely resistant as evidenced by the absence of clinical symptoms and brain lesions after the intracerebral test injections.

The results given in the text-figures show that the mice infected *in utero* carry virus for a longer period and in greater amounts than do those infected by contact. The great regularity in the persistence of virus in the blood of mice infected *in utero* (Text-fig. 1) is unrelated to the severity of the disease, which plays an important part in mice infected experimentally, as will be shown later. In the mice infected by contact shortly after birth (Text-fig. 2) the severity of the reaction likewise did not seem to influence the persistence of virus to any great extent. In another experiment, however, in which 2 newborn normal litters were exposed to suckling mice that had been injected intranasally with virus 6 days previously, the time of persistence of the virus appeared to be in proportion to the severity of the disease contracted by the exposed animals, as is shown in Table I. The blood of these mice was tested for virus $2\frac{1}{2}$ months after exposure.

Virus Content of Blood Fractions.—In previous tests made with blood from carrier mice the blood cells washed 3 times in large amounts of heparinized saline were either devoid of infectivity or

contained very little virus in comparison with the plasma. Similar observations were made with the blood of animals taken during the acute stage of the disease (3, 4). In three more recent tests with carrier blood the washed cells contained considerable amounts of virus, which in two experiments amounted to about one-tenth of the virus content of the plasma, while in the third test the washed cells and the plasma were about equally virulent.

TABLE I

Influence of the Severity of the Disease on the Persistence of Virus in Suckling Mice after Contact Infection

Litter No.	Mouse No.	Severity of disease	Result of test for virus in blood (2½ months after exposure)
1	1	+++	Positive
	2	+++	"
	3	+++	"
	4	+	"
	5	+	"
	6	0	Negative
	7	0	"
	8	0	"
2	9	+++	Positive
	10	+++	"
	11	+++	"
	12	+	"
	13	+	"
	14	+	Negative
	15	+	"
	16	±	"
	17	±	"
	18	±	"

Distribution of Virus in the Body of Carrier Mice.—It was of interest to determine whether the blood of carriers contained the bulk of the virus present in the body, or whether the high virus content of certain organs was responsible for the virus present in the circulation.

In the experiments presented in Table II the virus content of the blood was compared with that of certain tissues, and it can be seen that almost all the organs tested contained more virus than could be

TABLE II

Comparison of Virus Content of Blood and Organs of Old Carriers

Mouse No.	Date of test	Titer													
		Blood	Nasal turbinates	Submaxillary salivary glands	Lungs	Brain	Kidneys	Ovaries	Uterus	Testicles	Liver	Spleen	Submaxillary lymph nodes	Bronchial lymph nodes	Inguinal lymph nodes
	1937														
5, litter N	Dec. 13	10^{0}	?*	10^{-1}	10^{-1}	10^{-2}	10^{-3}+†	10^{-1}	10^{-2}		10^{-1}	10^{-1}			
2, " K	" 28	10^{0}	10^{-2}		10^{-2}		10^{-3}+								
	1938														
3, " N‡	Feb. 24	10^{-3}	10^{-6}+	10^{-6}+	10^{-6}+	10^{-6}+	10^{-6}+	10^{-6}+	10^{-6}+		10^{-6}+	10^{-6}+	10^{-6}+		
1, " G	Mar. 25	10^{-3}	10^{-5}	10^{-6}	10^{-6}	10^{-7}	10^{-6}			10^{-6}	10^{-5}	10^{-6}	10^{-7}+		
1, " M	Apr. 8	10^{-4}	10^{-7}				10^{-6}			10^{-6}			10^{-5}	10^{-5}	$>10^{-5}$

* The injected mice died of bacterial infection.

† Titration end-point perhaps not reached at this dilution.

‡ This animal showed beginning lymphatic leukemia at autopsy, the submaxillary lymph nodes being as large as small peas.

accounted for by their blood content. It is possible therefore that the virus in the blood comes from the infected tissues with which the blood comes in contact during circulation.

As reported before (1, 2) virus can be demonstrated with ease in the urine and nasal washings of naturally infected virus carriers. The same is true in the present experiment as is shown in Table III.

TABLE III

Presence of Virus in Urine and Nasal Washings from Old Carrier Mice

Mouse No.	Date of test	Result of pad injection in guinea pigs			
		Urine		Nasal washings	
	1937				
2, litter A	Nov. 2	10^0	D14*	10^0	D 14
	" 9	10^{-1}	D 15	10^{-1}	D 13
		10^{-2}	D 15	10^{-2}	D 14
		10^{-3}	D 27	10^{-3}	D 17
2, " B	Nov. 2	10^0	F, no S, I†	10^0	F, no S, I
	" 20	10^{-2}	" "	10^{-2}	" "
		10^{-3}	" "	10^{-3}	" "
3, " H	Nov. 2	10^0	" "	10^0	" "
	1938				
	Jan. 17	10^0	" "	10^0	" "
	1937				
2, " I	Nov. 2	10^0	" "	10^0	" "

* The inoculated guinea pig died in 14 days.

† The guinea pig showed fever, but no definite symptoms, and was immune to reinoculation with virus.

The virulence of the materials for guinea pigs varied considerably as in the previous experiments. Mouse 3, litter H, for instance, was presumably infected with the same strain of virus as mouse 2, litter A, to which it was exposed, and yet the virus discharged by the 2 mice differed markedly in virulence for guinea pigs. The consistency with which the virus from mouse 2, litter A, killed guinea pigs, and the strains from the other mice induced mild, febrile reactions supports the theory advanced before (5) that the differences observed

are mainly due to variations in the virulence of the virus and not to differences in the susceptibility of guinea pigs.

The virus present in the nasal secretions possibly originated in the nasal turbinates which contained considerable amounts of virus compared with the blood (see Table II), and the high virus content of the urine may have been due to that of the kidneys. Since the salivary glands and testicles were rich in virus, it can be assumed that the secretions of these glands may play a part in the transmission of the virus in exceptional cases. The relatively high virus content of the ovaries and uterus may be important for the infection of embryos, which so frequently occurs in pregnant females carrying virus.

The secretion and excretion of large amounts of virus, which occur over a long period of time, suggest that the virus continues to multiply in the tissues of carriers. If this were not the case, one would expect the virus content of certain tissues, for instance the kidneys and nasal turbinates, to become exhausted in due time. The multiplication of virus does not seem to be associated with an extensive breakdown of infected cells, since necrotic lesions are not frequent in the tissues of chronically infected mice. The lesions detected (1) are mostly inflammatory changes, in which mononuclear cells, particularly lymphocytes, predominate.

Virus Content of Mouse Blood during the Acute Stage of the Disease

A comparison has been made of the virus content of the blood of carrier mice with that of mice infected naturally or experimentally and bled during the acute stage of the disease.

The mice infected *in utero* (Table IV) came from 3 litters born in the infected colony. Mice 1 to 5 were bled on the 1st day of life, mouse 6 on the 5th day, and mice 7 to 10 on the 6th day. None of the mice appeared ill, but the growth rate of their litter mates not bled seemed slightly decreased in comparison with that of normal mice of the same age.

The mice infected by contact were 1 day of age when exposed to new-born mice injected intranasally with virus (mice 1 to 4) or to suckling mice infected *in utero* (mice 5 to 7). The mice were bled on the 17th (mice 1 to 3 and 5 to 7) or 21st (mouse 4) day of exposure, when they showed symptoms of the disease. Mouse 3 was bled twice, first on the 17th day, when it was only slightly sick, and again on the 21st day, when it appeared very ill.

Of the 4 new-born animals injected intranasally with virus, mice 1 to 3 were

bled on the 10th day after inoculation, and mouse 4 on the 13th day. The animals were sick at the time of bleeding.

The mice inoculated intravenously with virus were bled 6 to 8 days after injection, when they showed symptoms of the disease, and the blood of the mice

TABLE IV

Virus Content of Mouse Blood during the Acute Stage of the Disease

Mode of infection and age of mice	Maximum dilution of heart blood producing typical symptoms									
	Mouse No.									
	1	2	3	4	5	6	7	8	9	10
Intrauterine infection. Blood tested for virus at age of 1–6 days	10^{-4}	10^{-5}	10^{-4}	10^{-3}	10^{-5}	10^{-4}	10^{-4}	10^{-3}	10^{-5}	10^{-3}
Contact infection. Exposure to infected mice at age of 1 day. Blood tested for virus at age of 17–21 days	10^{-5}+*	10^{-4}	10^{-2} 10^{-5}	10^{-5}	10^{-5}	10^{-4}	10^{-4}			
Intranasal instillation of virus at age of 1 day. Blood tested 10–13 days later	10^{-4}	10^{-4}	10^{-6}	10^{-3}						
Intravenous inoculation in 5-wk.-old mice. Tested 6–8 days after injection	$>10^{-3}$	$>10^{-3}$	10^{-4}	10^{-5}	10^{-5}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}
Intracerebral inoculation in 5-wk.-old mice. Tested 7–9 days after injection	$>10^{-3}$	$>10^{-3}$	$>10^{-3}$	10^{-2}						

* + = titration end-point not reached at this dilution.

inoculated intracerebrally was taken on the 7th to 9th day, when they showed characteristic convulsions.

The stock strain of virus described below was used for all experimental infections in this experiment. Blood from anesthetized suckling mice was obtained by heart puncture, 0.02 or 0.05 cc. blood (according to the size of the animals) being

withdrawn with a 0.25 cc. syringe and a 27 gage needle. The blood was mixed immediately with 1.98 or 4.95 cc. heparinized saline, and from this 10^{-2} dilution further dilutions were made. This method is not accurate, but colorimetric tests showed the inaccuracy to be negligible in view of the crude method of titration used. The highest decimal dilution producing typical symptoms in a mouse injected intracerebrally was regarded as the titration end-point.

The suckling mice infected *in utero* or by contact are most suitable for a comparison with the carrier mice recorded in Text-figs. 1 and 2. This shows that the average virus content of the blood (34,300 M.I.D. per 0.05 cc. heart blood) of the 10 mice infected *in utero* (Table IV) is only about twice as great as that of the old carriers (19,000 M.I.D. per 0.05 cc. blood) infected in the same manner and recorded in Text-fig. 1. Of the carriers only those animals were considered in which the titration end-point of the blood was reached. With the mice infected by contact soon after birth the difference in virus content between the chronically and acutely infected animals is much greater.

In the groups of suckling mice infected either by contact or by intranasal instillation of virus (Table IV) the virus content of the blood was greater in animals that were very sick than in those which showed a comparatively slight reaction, and the same was true for the mice injected intravenously. The blood of the mice inoculated intracerebrally with virus was much less infectious than that of the mice infected in other ways. The reason for this difference is not known.

The Influence of the Mode of Experimental Infection on the Persistence of Virus in the Blood of 5 to 6-Week-Old Mice

The three strains of virus used were identical immunologically but differed in their pathogenicity for mice.

The "stock" strain has been maintained by natural passage in the infected stock since 1934. The thoracic and abdominal organs of suckling mice were used as sources of virus. All of the normal mice injected intracerebrally with such virus showed characteristic symptoms 6 to 8 days after inoculation, but the rate of mortality varied between 50 and 100 per cent with different virus suspensions. The majority of the mice inoculated intravenously or intraperitoneally became sick, the mortality varying within wide limits. Subcutaneous inoculation always failed to cause symptoms, but was followed by circulation of

virus and immunity to intracerebral injection with virus. Intranasal instillation[1] rarely produced symptoms. The disease so induced was never fatal and immunized mice against subsequent intracerebral inoculation with virus.

The second strain was the guinea pig passage strain B described before (5) and maintained by serial pad passages in guinea pigs, using brain suspension as inoculum. The virus had undergone 15 to 18 serial transfers. Its virulence for mice is comparable to that of the stock strain, except that it more often causes disease after intranasal instillation.

The third strain was the mouse passage strain B also mentioned before (5). It was modified by serial brain-to-brain transfers in mice. The virus used in the present experiments had been passed through 40 to 45 mice. It is highly virulent when injected intracerebrally but has failed to produce symptoms after inoculation by other routes. Intravenous, intraperitoneal, subcutaneous, or intranasal injection was always followed by immunity, while a small percentage of the mice inoculated intranasally failed to become resistant.

A summary of the data on the pathogenicity of the three strains is given in Table V.

In the following experiments a number of mice were injected by different routes with each strain, and their blood was tested for virus at various periods of time after inoculation. In order to correlate the virus content of the blood with the immunity, the mice that survived the disease as well as the bleedings were tested for immunity 2 to 3 weeks after the blood test, except one group of mice inoculated intravenously with mouse passage virus and tested for immunity on the 6th day, immediately after the blood test. A suitable number of normal control mice was used in every case.

The inoculum consisted of the supernatant of a 10 per cent suspension, centrifuged for 5 minutes at about 1600 R.P.M., of the thoracic and visceral organs of suckling mice from the infected colony (stock strain), the supernatant of a 10 per cent guinea pig brain suspension (guinea pig passage strain), or the supernatant of a 10 per cent mouse brain suspension (mouse passage strain). The dosage on intravenous, intraperitoneal, or subcutaneous inoculation was 0.25 cc., that on intranasal instillation 0.05 cc. Since homologous brain extract is very toxic when injected intravenously in mice, the mice inoculated by this route with the mouse passage strain received 0.25 cc. supernatant of a 10 per cent suspension of the brain of a guinea pig that was killed during fever on the 6th day after intracerebral inoculation with mouse passage virus.

The essential details of the experiment are presented in Table VI which shows the marked differences obtained with different modes of

[1] 0.05 cc. supernatant of a tissue suspension was dropped on the nostrils of an anesthetized mouse with a 0.25 cc. syringe and a 27 gage needle. The material was promptly inhaled, and the results obtained by this method were much more consistent than those of the procedure employed before (6).

infection and different strains of virus. It can be seen that the duration of the infection in general corresponded to the severity of the disease. The more severe the reaction of the mice was, the longer the virus persisted in their blood.

The results obtained with different strains of virus, however, are not entirely explained by the differences in the severity of the disease induced by them. Subcutaneous inoculation of the guinea pig passage virus, for instance, failed to cause a visible reaction in any of the

TABLE V

Virulence of Three Strains of Choriomeningitis Virus for 5 to 6-Week-Old Mice

Strain of virus	Route of inoculation				
	Intra-cerebral	Intrave-nous	Intraperi-toneal	Subcuta-neous	Intranasal
Stock	+ (50–100)*	± (0–60)	± (0–43)	– (0)	∓ (0)
Guinea pig passage	+ (70–80)	+ ?†	+ (20)	– (0)	± (0)
Mouse passage	+ (80–100)	– (0)	– (0)	– (0)	– (0)

\+ = all injected mice became sick.
± = over 50 per cent of the injected mice became sick.
∓ = less than 50 per cent of the injected mice became sick.
– = all injected mice showed no symptoms.

* The percentage of mice that died is given in parentheses. The mice inoculated intracerebrally received more than 10 M.I.D. of virus. Smaller amounts often produce inapparent infection followed by immunity.

† The number of mice injected intravenously with guinea pig passage virus was too small to determine the average rate of mortality.

12 mice injected but created a high percentage of carriers. The mouse passage strain, on the other hand, was not demonstrable in the blood of 6 mice recovered from severe convulsions caused by intracerebral infection and bled about 1 month after inoculation (these tests are not recorded in the table; they were made whenever mice recovered from intracerebral infection were available). It seems therefore as if certain properties of the virus distinct from its pathogenicity accounted for these results.

TABLE VI

Persistence of Virus in the Blood of 5 to 6-Week-Old Mice Injected by Different Routes with Different Strains of Virus

Strain of virus	Route of inoculation	Severity of disease	Test for virus in blood			Degree of immunity		
			Time after injection	Virus carriers: No.	Virus carriers: Percentage	Complete	Partial (accelerated, non-fatal reaction)	None
			days					
Stock	iv	++ (1)*	160	9/15†	60	14/14†	0	0
	ip	+++ (3)	160	7/12	58.3	10/10	0	0
	in	− (0)	160	2/14	14.3	9/13	4	0
	sc	− (0)	160	0/12	0	0/12	12	0
"	ip	± (0)	60	2/13	15.4	9/12	3	0
	in	− (0)	60	0/12	0	5/10	4	1‡
	sc	− (0)	60	0/12	0	1/11	10	0
Guinea pig passage	ip	++ (4)	60	9/12	75	10/10	0	0
	"	+ (0)	60	3/8	37.5	8/8	0	0
	sc	− (0)	60	8/12	66.7	9/10	1	0
Mouse passage	iv	− (0)	45	0/13	0	0/13	12	1‡
	ip	− (0)	6	7/12	58.3	11/11	0	0
	"	− (0)	30	0/12	0	9/10	1	0
	in	− (0)	30	0/10	0	5/9	2	2§

iv = intravenously.
ip = intraperitoneally.
in = intranasally.
sc = subcutaneously.

* The number of mice that died from the disease is given in parentheses.

† The numerator represents the number of mice with either circulating virus or complete immunity; the denominator indicates the number of mice tested.

‡ Dead on the 2nd day after intracerebral inoculation.

§ " " " 7th " " " "

Tests made from time to time with the urine and nasal washings of some of the mice recorded in Table VI, using guinea pigs as test animals, gave results similar to those of the blood tests.

The different degrees of immunity observed in the mice require some explanation. If an animal showed no visible reaction whatever

to the intracerebral test injection during a 2 weeks' period of observation, its immunity was considered as complete. "Partial immunity" manifested itself in an accelerated but non-fatal reaction to the test inoculation. Such partially immune mice would show marked symptoms of malaise, inappetence, loss of weight, ruffled fur, as well as striking tremors and jerky motions of the hind extremities when lifted by the tail, on the 2nd or 3rd day after the test injection. Convulsions have not been noted, and the animals as a rule made a quick recovery, appearing practically normal on the 5th or 6th day after inoculation, when the normal controls injected in the same manner began to show the first symptoms. Histological examination of the brains of mice killed during the accelerated reaction on the 2nd day after inoculation revealed a marked meningo-encephalitis, which could hardly be distinguished from that presented by control mice killed at the height of the disease on the 6th or 7th day after injection, except that the tendency to cerebral hemorrhages was greater in the former animals. The brains of control mice killed on the 2nd day after inoculation showed only traces of meningitis, if any. It is believed that the accelerated reaction is due to the allergic state of the mice with a waning immunity.

As can be seen from Table VI, the number of partially immune, allergic mice was greatest in those groups that contained few or no carriers, while the mice of other lots with a high percentage of carriers as a rule were completely immune. Particularly noteworthy is the fact that all of the mice with a partial immunity gave a negative blood test.

This could make it appear that the immunity of mice depended upon the presence of active virus in the body. However, numerous other observations do not justify this conclusion. In the first place, we have repeatedly encountered mice with a high degree of resistance in whose bodies no virus was detected (1). The problem was reinvestigated recently, when the results given in Table VI were obtained, and the experiments performed provided additional evidence suggesting that there is an immunity independent of the presence of active virus in the organism. For instance, when mice that had shown a severe accelerated reaction after the intracerebral test injection with mouse passage virus were reinoculated with the same

strain by the same route 1 month later, they failed to show any reaction. Some such mice killed 16 days after the last inoculation carried no demonstrable virus in their brains or in any of their blood fractions. It is unlikely that the presence of virus was masked by antibodies, since these were either absent from the blood and from a concentrated brain extract, or possibly present in very small amounts, which could hardly have been effective. Other tests gave similar results.

The Influence of Age on the Persistence of Virus in the Blood after Intranasal Instillation

It has been reported (7, 4, 6) that intranasal instillation of choriomeningitis virus does not cause illness in mice but immunizes them to intracerebral injection with virus. In more recent experiments, however, it has been found that a symptomless infection occurs only in mature mice (5 weeks of age or older) inoculated intranasally with certain strains of virus, while immature mice as a rule become sick and often die.

Some of the inoculations are recorded in Table VII to show the difference in reaction of mice of different ages. Suckling mice received 2 drops of the supernatant of a virulent 10 per cent mouse or guinea pig brain suspension through a 27 gage needle; 2 to 3-week-old mice received 3 drops; while the mice 5 weeks of age or older were given 0.05 cc.

The stock strain caused no signs of illness in the great majority of the mature mice, while young mice as a rule became sick and many of them died. The guinea pig passage virus was the most virulent and even produced a relatively mild, non-fatal disease in mature mice. Intranasal instillation of the mouse passage virus was effective only in very young animals.

The symptoms produced in mature mice were those of general malaise and labored respiration. They did not point to an involvement of the central nervous system. The same was generally true for immature animals, which showed visceral lesions without meningitis or with only traces of it. A few of the more than 100 young mice injected, however, showed characteristic convulsions that are otherwise seen only in mice injected intracerebrally. In 2 to 3-week-old

mice the guinea pig passage virus frequently produced serous pleuritis ending in respiratory failure.

A number of mice of different ages injected intranasally with stock or mouse passage virus were kept for a period of time after recovery and then tested for circulating virus. All mice, including those very sick for several weeks after inoculation, had completely recovered at

TABLE VII

Susceptibility of Mice of Different Ages to Intranasal Injection with Virus

Strain of virus	Age of mice	No. of mice injected	Result		
			No. of mice which		
			Showed no definite symptoms	Became sick and recovered	Died
Stock	1 day	18	0	13 (+++)*	5
	2–3 wks.	12	4	4 (++)	4
	5 "	12	12	0	0
	6–7 mos.	12	12	0	0
	2 wks.	14	0	5 (+++)	9
	5 "	14	3	11 (±)	0
	2 "	26	0	19 (+++)	7
	5 "	9	8	1 (+)	0
Guinea pig passage	1 day	16	0	2 (+++)	14
	7 days	14	0	4 (+++)	10
	2–3 wks.	20	0	7 (++)	13
	5 "	10	0	10 (+)	0
	9–10 "	10	0	10 (+)	0
Mouse passage	1 day	41	3	9 (+++)	29
	12–14 days	12	12	0	0
	5 wks.	12	12	0	0

* The number of + signs indicates the average severity of the disease.

the time of the blood tests and could not be differentiated from uninfected animals.

The results recorded in Table VIII show that the duration of the carrier state differed markedly with the age at the time of injection. The younger the mice were, the longer they carried virus in the blood. Very young mice infected with the stock strain became carriers more frequently than those infected with mouse passage virus.

The Influence of Age on the Persistence of Virus after Contact Infection

The disease induced in mice of different ages by contact infection is similar to that produced by intranasal instillation of virus in that very young mice often become sick, while the disease in mature ones invariably is subclinical. The duration of the infection in mice infected by contact is also comparable to that in mice injected intranasally with virus. As has been shown in Text-fig. 2, many young mice infected by contact carry virus in the blood for long periods of time,

TABLE VIII

Persistence of Virus in the Blood of Intranasally Injected Mice of Different Ages

Strain of virus	Age of mice when injected	Severity of disease	Test for virus in blood		
			Time after injection	Mice with virulent blood	
				Number	Percentage
			days		
Stock	1 day	+++	160	12/12	100
			250	6/6*	100
	1 wk.	+++	160	7/7	100
	5 wks.	±	160	2/14	14.3
"	1 day	+++	60	13/13	100
	2–3 wks.	++	60	5/8	62.5
	5 "	–	60	0/12	0
Mouse passage	1 day	+++	60	4/7	57.1
	12–14 days	–	30	0/8	0

* These 6 mice were among the 12 animals tested on the 160th day after inoculation.

whereas in mature mice infected by contact the virus circulates for 3 weeks at the most. This can be concluded from the data on other experiments on contact infection.

DISCUSSION

Among the factors influencing the persistence of virus after natural infection the age of the mice appears to be the most important one. The more immature the mouse tissues are at the time of infection, the more regularly the virus persists in them. This is indicated by

the fact that the virus remained longer or in greater amount in the blood of mice infected *in utero* than in those infected by contact during the first 3 weeks of life. The severity of the disease, which appears to be the determining factor in mature mice infected experimentally, was of minor importance compared with the age factor.

In the case of the mice of different ages infected by contact or by intranasal instillation of virus the age at the time of infection may likewise be important, but its effect can be less clearly differentiated from that of the severity of the disease, which was greatest in very young mice. These continued to carry the virus for longer periods of time than older mice that showed a slighter reaction.

In some groups of suckling mice infected by contact, as well as in mature mice infected experimentally, the duration of the infection corresponded to the severity of the symptoms which the infected animals had shown. The parallelism recalls the experiments of Beller and Biermann (8) with hog cholera virus, in which swine vaccinated by the simultaneous method discharged virus with the urine longer when they had shown a severe reaction than when the reaction had been entirely absent or very mild.

The differences in the persistence of strains of virus may be due to a combination of two factors, namely, the severity of the disease produced, and a property of the virus itself distinct from its pathogenicity. This is also suggested by the comparatively small percentage of carriers detected among the mice inoculated intranasally with the mouse passage virus during the first 12 days of life, which is in contrast to the high percentage of carriers created by the stock strain.

The high virus content of the blood and certain organs of carrier mice, particularly those infected *in utero*, is of interest with regard to the mechanism of their immunity. It is very unlikely that protective antibodies play an essential part in this immunity, because the average virus content of the blood of carriers infected *in utero* taken at the age of about 1 year is only slightly less than that of mice similarly infected and bled during the 1st week of life. If antibodies were fixed in the tissues, as Bedson (9) suggested, one would not expect to demonstrate such great amounts of virus in them as have been detected. It rather appears that the susceptible cells

of the body are infected in such animals, and that cells once infected with virus cannot be reinfected. This assumption is supported by the fact that new-born mice infected *in utero* are already immune to intranasal instillation of highly virulent virus, which is fatal to many of the new-born control mice and produces a severe disease in those which survive.

The observations of Hoskins (10) and of Findlay and MacCallum (11) on the interference phenomenon in yellow fever are of great interest in this connection. These authors have shown that monkeys infected simultaneously with viscerotropic and neurotropic yellow fever virus often survive, while monkeys inoculated with viscerotropic virus alone almost always die. This effect could even be demonstrated when the neurotropic virus was given up to 20 hours after the viscerotropic strain, and did not appear to be due to a precocious formation of antibodies (11). It seems therefore that cells once infected by the neurotropic virus are refractory to the viscerotropic strain. It is also interesting to note the parallelism between the immunity of carrier mice to choriomeningitis and that of plants to tobacco ring spot virus. Immune plants are always carriers of virus, although they show no signs of disease, but their virus content is about 5 times less than that of diseased plants that show symptoms (12).

If no other data were available than those given in the text-figures and in Tables II and VI, one might conclude that the immunity of mice to choriomeningitis was always an infection immunity. However, some observations reported before (1), as well as the results obtained with mice injected repeatedly with mouse passage virus, in whose blood and brains no virus was demonstrated, fail to justify this conclusion. The mechanism of the immunity of such mice has recently been investigated. While the sera of some animals may possibly have contained minute amounts of protective antibodies, the results obtained with other sera as well as with brain extracts from immune mice were negative. It is tentatively concluded for the present that the immunity of such mice is not an infection immunity, nor due solely to protective antibodies circulating in the blood or fixed in the tissue spaces. It rather appears to be linked in some way with the tissues.

It remains for future studies to determine whether the two kinds of immunity observed really differ, or whether the same fundamental mechanism underlies both of them.

SUMMARY AND CONCLUSIONS

Mice infected *in utero* continued to carry choriomeningitis virus in the blood more regularly and in greater amount than suckling mice infected by contact. This result may be due to the difference in tissue maturity at the time of infection: the more immature the tissues are when infected, the longer the virus appears to persist in them after maturation. A similar result was obtained with mice of different ages infected either by contact or by intranasal instillation of virus, in that the carrier state lasted longer in the younger animals. This cannot be attributed entirely to the difference in age, however, since young mice as a rule showed more severe symptoms than mature animals. It is possible, therefore, that the difference in the severity of the disease accounted in part for that in the duration of the infection.

In mature mice infected experimentally as well as in some of the suckling mice infected by contact the severity of the disease was the determining factor, the infection persisting longest in those animals that showed the most severe reaction.

The character of the virus used also appeared to influence the persistence of the virus in the blood. A strain of virus isolated in 1935 from an infected stock mouse and modified by intracerebral passage in mice (5) disappeared from the circulation more rapidly than the stock strain maintained by natural passage in the infected mouse stock. The guinea pig passage strain, however, which was obtained from the same mouse as the mouse passage virus but passed through guinea pigs by pad inoculation, persisted in the blood more frequently than the stock strain.

Carrier mice without exception had a high degree of immunity to intracerebral injection with virus, while other animals once infected but no longer carrying detectable amounts of virus in the blood often showed an incomplete immunity that manifested itself in an accelerated, non-fatal reaction, presumably of an allergic nature. This observation does not prove, however, that the immunity always is an

"infection immunity," since a high degree of resistance not associated with detectable amounts of virus in the blood and brain was produced by repeated injections with the mouse passage strain.

Since the blood and the tissues of old carriers often contain large amounts of virus, it is very unlikely that their immunity is due to protective antibodies circulating in the blood or fixed in the tissue spaces. It rather appears that the susceptible cells of such animals are infected and that cells occupied by actively multiplying virus cannot be reinfected. The mechanism of this infection immunity as well as the immunity apparently not associated with infection requires further study.

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