THE INFLUENCE OF THE AGE OF THE HOST ON LOCAL VIRUS MULTIPLICATION AND ON THE RESISTANCE TO VIRUS INFECTIONS

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(With 4 Figures in the Text)

INTRODUCTION

Susceptibility to many neurotropic virus infections varies inversely with the age of the host, particularly when the virus is given by a peripheral route rather than directly into the brain (Sigel, 1952).

In some cases the greater susceptibility of young animals may be due to their inability to respond to the inoculum with sufficient antibody formation to neutralize the virus before being themselves overwhelmed by infection (Morgan, 1941). However, in other cases it is known that resistance can develop in growing animals without either any previous exposure to infection, or any specific virus-neutralizing antibody in the blood (Olitsky, Sabin & Cox, 1936). The wide variations (Sigel, 1952) encountered in the rate and extent of the development of resistance to different viruses, and to the same virus injected by different routes, also suggest that the immune response is not sufficient to account for the whole of the data on age resistance.

It is therefore likely that some progressive alteration with age takes place in the properties of the host cell itself, affecting its mode of interaction with the invading virus. One possibility is that the virus can gain entry to the cells of both young and old hosts with equal facility, but having done so can only multiply in the former. This view is at first sight supported by the observation of MacDonald & Sanders (1959), who, in the course of investigating the development of resistance to the GDVII strain of mouse encephalomyelitis, were able to detect considerable multiplication of small inocula of virus in the muscles of young susceptible mice (see also Rustigian & Pappenheimer, 1949), but not of older resistant animals. However, in the present paper, which describes an investigation into the development, with age, of resistance in mice to the virus of encephalomyocarditis administered by various routes, and to two strains of Coxsackie virus, we bring forward evidence which renders this interpretation untenable. While confirming the observation of MacDonald & Sanders (1959) for all three viruses studied, we consider that the experiments reported here indicate that it is the accessibility of the

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cells to virus which alters with the age of the host, rather than the ability of these cells to support virus multiplication. Failure to demonstrate multiplication in older animals would then be attributable to failure of the virus to obtain entry into the cells, without which multiplication is of course impossible.

MATERIALS AND METHODS

Viruses

The four viruses used in the present work were the virus of encephalomyocarditis (Helvig & Schmidt, 1945; Warren, Smadel & Russ, 1949), the GDVII strain of mouse encephalomyelitis (Theiler & Gard, 1940), and the Texas (Group A, antigenic type 4) (Dalldorf, Sickles, Plager & Gifford, 1949), and Connecticut-5 (Group B, antigenic type 1) (Melnick, Shaw & Curnen, 1949) strains of the Coxsackie group of viruses. These viruses will henceforward be referred to as EMC, GDVII, Texas and Conn-5.

Stock suspensions of the viruses were made by homogenizing infected mouse brains in a chilled Waring blendor for 5 min. in the case of EMC and GDVII, and infected whole baby mice, skinned and eviscerated, in a glass homogenizer in the case of the Coxsackie viruses. 10% (v/v) normal rabbit serum in isotonic phosphate-buffered saline, pH 7·2, was used as diluent for the EMC and Coxsackie viruses, distilled water for the GDVII virus. The 50% lethal dilutions of the EMC and GDVII stocks, determined by intracerebral titration (0·03 ml. inocula, eight mice per tenfold dilution), were $10^{-8\cdot4}$ and $10^{-8\cdot0}$, respectively. The 50% lethal dilution of both Coxsackie pools, titrated intracerebrally in 5-day-old mice (0·01 ml. inocula, five mice per dilution) was $10^{-6\cdot6}$; 50% end-points were calculated by the method of Reed & Muench (1938) throughout.

The stock suspensions were stored in a dry-ice cabinet, and did not alter significantly in infectivity over the period during which the experiments described were carried out.

Intramuscular inoculations of virus were made into the calf muscles of the right hind leg and intracerebral inoculations into the left cerebral hemisphere. Mice to be fed with a virus suspension were held on their backs, and the virus dripped into their mouths through a hypodermic needle; their tails were pinched and their palates tickled until they swallowed the dose.

Mice

The CBA inbred strain of mice was used for the work on EMC age resistance, and albino mice from a dealer (accredited by the Laboratory Animals Bureau) for the Coxsackie experiments. For the work on virus multiplication, mice from the CBA and C57BL inbred strains were used. For the experiments on GDVII multiplication, mice were used from a line free from the intestinal strain of mouse encephalomyelitis virus (TO strain), in order to avoid the complicating factor of antibody to this strain (von Magnus & von Magnus, 1949).

Since births were only recorded once daily, the ages of the mice used may be anything up to 24 hr. greater than that given. The day on which a litter was found was counted as day 0.

RESULTS

Influence of age on susceptibility to virus infection

Since the infectivity of the stock virus suspensions remained constant, the susceptibility of mice of a given age to a given virus administered by a given route may be expressed as the 50 % lethal or paralytic dilution of the stock, determined by appropriate titrations (five to twelve mice per tenfold dilution). Symptoms were recorded for 21 days after inoculation.

(*a*) *Encephalomyocarditis virus* (*EMC*)

Mice of all ages are equally susceptible to EMC virus injected into the brain. The results of giving virus (0·03 ml. inocula) by three other routes—intraperitoneal, intramuscular and oral—are shown in Fig. 1.

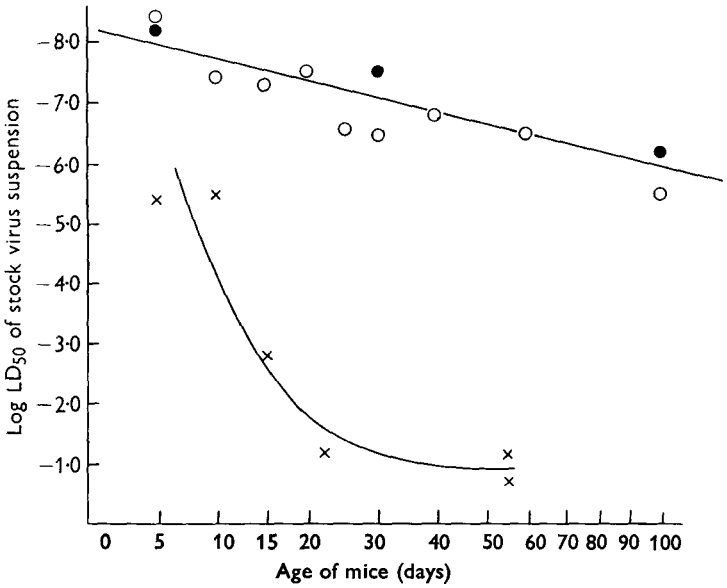

Fig. 1. Development, with age, of resistance to encephalomyocarditis virus administered by various routes. (The stock virus suspensions used had an intracerebral titre of $10^{-8\cdot4}$, which was independent of the age of the mice used for the titration.) ○, Intramuscular; ●, intraperitoneal; ×, oral.

It will be seen that mice develop with age a resistance to EMC virus administered by all three of these peripheral routes, although initially almost as susceptible by these routes as they are to virus given intracerebrally. However, at no age do any of the paralysed mice recover. Resistance to intramuscular injection developed steadily throughout the period investigated, and by the time the mice were 3 months old, nearly 1000 times as much virus was needed to kill them if injected into a muscle ($LD_{50} = 10^{-5\cdot6}$), as was necessary if injected directly into the brain ($LD_{50} = 10^{-8\cdot4}$). The results for intraperitoneal inoculation do not differ significantly from those for intramuscular inoculation. The resistance to EMC virus given by mouth develops extremely rapidly between 10 and 20 days of age, and

22-day-old mice are almost completely resistant to feeding with EMC. Culbertson (1939) found that a striking change took place between 10 and 20 days of age in the ability of rats to be passively immunized by the oral administration of specific antiserum. Both these changes might be due to a decline with age in the permeability of the gut to proteins, perhaps related to the change in feeding habits which occurs around the 15th day, when the mice begin to eat solid food.

(*b*) *Coxsackie virus, Group A, Texas strain*

Group A Coxsackie virus injected intracerebrally kills new-born mice and hamsters, but does not affect adults (Dalldorf *et al.* 1949). Goldblum (cited by Melnick & Curnen, 1952) noted that, in mice, resistance was virtually complete by the age of 3 weeks. Throughout the age range lesions appear to be confined to striated muscle.

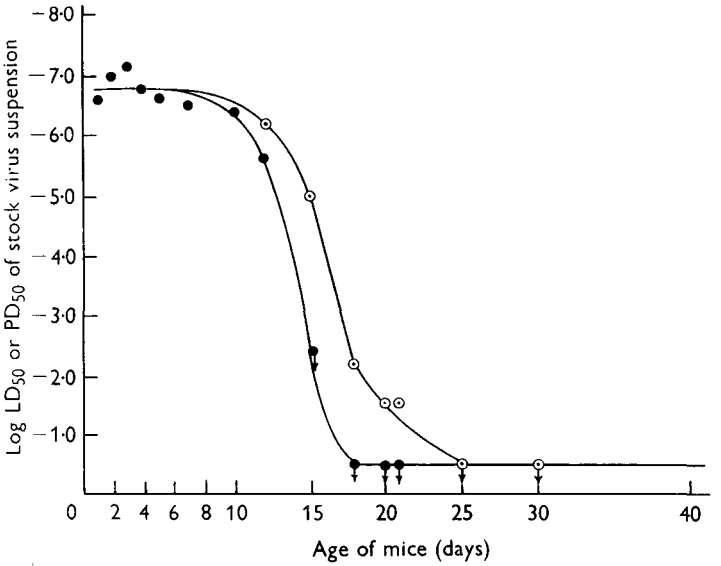

Fig. 2. Development, with age, of resistance to Coxsackie virus, Group A Texas strain (intramuscular inoculation). ● = 50 % lethal dilution; ⊙ = 50 % paralytic dilution.

The results of the intramuscular injection of 0·01 ml. inocula of Texas virus into mice of different ages are shown in Fig. 2, which gives both the 50 % lethal and the 50 % paralytic dilutions. The greatest increase in resistance occurs between the ages of 12 and 20 days, and after 21 days the mice are completely resistant to the Texas strain of Coxsackie given by this route. Similar results were obtained with intracerebral inoculation, 5-day-old mice being highly susceptible ($LD_{50} = 10^{-6\cdot2}$) and 30-day-old mice completely resistant.

Up to 12 days all the paralysed mice die, so that the 50 % paralytic dilution and the 50 % lethal dilution coincide. However, throughout this early period, the interval elapsing between the appearance of symptoms and death becomes progressively longer. After 12 days some of the mice injected with the higher dilutions of virus become paralysed but subsequently recover, so that the 50 %

paralytic dilution is higher than the 50 % lethal dilution, and by about 18 days *all* the paralysed mice recover. The function proposed by Gard (1940) in a slightly different context can be used to express quantitatively the lengthening with age of the period of paralysis, since it takes into account the recovery of some of the paralysed mice. The function is

$$\frac{1}{T} = \frac{1/t_1 + 1/t_2 + 1/t_3 + \ldots + 1/t_N}{N},$$

where $t_1 \ldots t_N$ are the individual intervals between onset of paralysis and death, and N is the number of mice inoculated. In the case of a mouse recovering from paralysis, t is taken as infinite and its reciprocal is 0. This function, the 'reciprocal of mean survival time' in Fig. 3, is actually the reciprocal of the harmonic mean (T) of the interval between onset of paralysis and death.

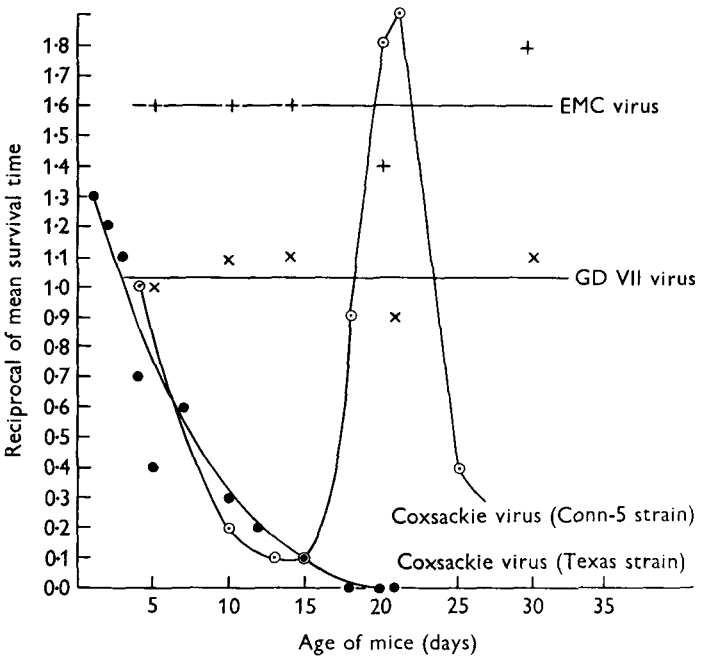

Fig. 3. Variation of mean survival time with age for four strains of virus.

Fig. 3 shows $1/T$ plotted against the age of the mice in days, for intramuscular titrations of the two Coxsackie strains, EMC and the GD VII strain of encephalomyelitis. With EMC and GD VII virus, where none of the paralysed mice recover, the survival time is short and does not alter significantly with age. With Texas virus, the reciprocal of the harmonic mean of survival time falls rapidly from birth onwards, reaching zero at about 18 days—that is, the paralysed mice survive progressively longer as they grow older, until at 18 days they all recover.

(*c*) *Coxsackie virus, Group B, Connecticut-5 strain*

Group B Coxsackie virus, like the Group A strains, only produces encephalitis and subsequent death in very young mice (Melnick *et al.* 1949). However, with Group B viruses the relation between age of host and effect of virus is complicated

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by the changes in tissue affinity of the virus in hosts of different ages. Thus adult mice inoculated with the same virus develop pancreatitis but no encephalitis. Pappenheimer, Kunz & Richardson (1951) showed that, unlike the Group A strains, Group B virus causes severe pancreatic lesions in mice younger than 7 days or older than 20 days. Such lesions have also been detected in new-born mice by Godman, Bunting & Melnick (1952).

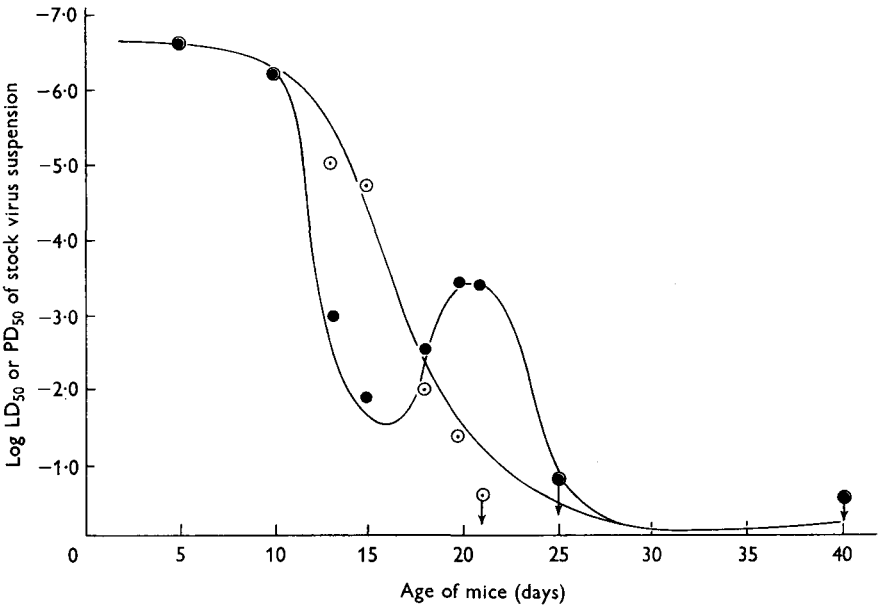

Fig. 4. Development, with age, of resistance to Coxsackie virus, Group B, Conn-5 strain (intramuscular inoculation). ● = 50 % lethal dilution; ⊙ = 50 % paralytic dilution.

Fig. 4 shows the results obtained from intramuscular inoculation of 0·01 ml. of serial dilutions of Conn-5 virus into mice of different ages. The general form of the curve is similar to that obtained for the Texas strain. A sharp fall in susceptibility again occurs between 12 and 20 days; and the curve for the 50 % paralytic dilution diverges from that for the 50 % lethal dilution, so that some of the mice on the higher dilutions become paralysed but later recover. The same progressive lengthening of duration of paralysis with age is also encountered. With intracerebral inoculation (0·01 ml. dose), 5-day-old mice were again highly susceptible ($LD_{50} = 10^{-6·2}$), and 30-day-old mice completely resistant.

The striking difference between the Texas and Conn-5 curves lies in the sharp peak in the Conn-5 curve for 50 % lethal dilution, which occurs around 20 days, coinciding with the time of weaning. This means that mice are *more* susceptible to Conn-5 virus injected intramuscularly at 20 days than they are at 15 days. However, the increased deaths at higher dilutions which occur at 20 days are not preceded by paralysis as are the deaths in younger mice, and all deaths at any age in mice injected with the Texas strain; and there is therefore no corresponding rise in the curve for 50 % paralytic dilution. The deaths are either sudden, with no previous external symptoms, or preceded by a period of generalized sickness, the

mice losing weight and their coats becoming 'staring'. Pappenheimer *et al.* (1951) also observed an unusually high number of deaths in their 22-day-old group. Possibly the pancreatic lesions produced by the virus more often prove fatal to mice at this stage of development on account of the change of diet associated with weaning, which occurs at about 21 days in the mouse. The striking pancreatic lesions, also seen in older mice, only rarely prove fatal (Pappenheimer *et al.* 1951; Dr A. Vizoso, personal communication).

The peak at about 21 days in the reciprocal survival time curve (Fig. 3) for the Conn-5 strain corresponds to this period during which the mice died suddenly, with little or no preliminary period of paralysis.

Virus multiplication following the injection of small amounts of virus

Virus multiplication in the brain and calf muscles was studied. A very small inoculum, 0·01 ml., was used throughout in order to localize the inoculum as far as possible at the site of injection. Forty-eight hours after inoculation the mice were killed, and the virus content of homogenates of infected tissue was estimated by intracerebral titration in the case of EMC and GD VII viruses, and intramuscular titration in 5-day-old mice for the two Coxsackie strains.

A comparison of total virus injected with virus present in the tissue after 48 hr. gives a rough estimate of virus multiplication. Since the inoculum never remains wholly localized in the injected tissue, and no account is taken of any virus 'eclipse', the amount of virus multiplication which takes place is an underestimate throughout.

(*a*) *Texas and Conn-5 strains of Coxsackie virus*

Table 1 shows that when a 10^{-5} dilution of the stock suspension of either Coxsackie strain (i.e. about forty 5-day-old intracerebral LD_{50}'s per mouse) was

Table 1. *Multiplication of two strains of Coxsackie virus in the brain and muscles of mice of different ages. Each mouse received forty 5-day-old intracerebral* LD_{50}*'s*

Strain of virus	Age of mice (days)	Tissue	Total virus injected (in 5-day-old intracerebral LD_{50}'s)	Total virus content of tissues after 48 hr. (in 5-day-old intracerebral LD_{50}'s)	Apparent virus multiplication
Texas	5	Brain	$1{\cdot}6 \times 10^2$	3×10^9	2×10^7
		Muscle	$2{\cdot}0 \times 10^2$	6×10^7	3×10^5
	20	Brain	8×10^1	2×10^4	2×10^2
		Muscle	8×10^1	2×10^3	2×10^1
	30	Brain	8×10^1	2×10^3	2×10^1
		Muscle	8×10^1	$\leqslant 1 \times 10^2$	$\leqslant 1$
Conn-5	5	Brain	$1{\cdot}6 \times 10^2$	3×10^9	2×10^7
		Muscle	$1{\cdot}6 \times 10^2$	2×10^8	1×10^6
	30	Brain	8×10^1	$\leqslant 3 \times 10^2$	$\leqslant 4$
		Muscle	$1{\cdot}6 \times 10^2$	$\leqslant 1 \times 10^2$	$\leqslant 1$

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injected into the brain or muscle of groups of 5-day-old mice, 10^5–10^7 times as much virus was present in the tissue after 48 hr. as had been injected initially. This multiplication factor declined with age, and no significant degree of multiplication of either strain could be detected in the brain or muscle of 30-day-old mice.

(*b*) *EMC virus*

Multiplication of EMC virus in the calf muscles of groups of mice of different ages was studied, using an inoculum containing about eight intracerebral LD_{50}'s per muscle. Table 2 shows that, in mice up to 20 days old, 10^4–10^5 times as much virus could be extracted at the end of the 48 hr. as had been injected initially. In mice older than 30 days, no significant virus multiplication could be detected by this method.

Table 2. *Multiplication of EMC virus in the muscles of mice of different ages. Each mouse received approximately eight intracerebral* LD_{50}*'s*

Age of mice (days)	Total virus injected (in intracerebral LD_{50}'s)	Total virus content of muscles after 48 hr. (in intracerebral LD_{50}'s)	Apparent virus multiplication
5	5×10^1	1×10^6	2×10^4
15	$1{\cdot}1 \times 10^2$	1×10^7	9×10^4
20	8×10^1	1×10^7	1×10^5
25	6×10^1	$\leqslant 1 \times 10^2$	$\leqslant 2$
30	6×10^1	3×10^3	5×10^1
40	$1{\cdot}2 \times 10^2$	$\leqslant 3 \times 10^2$	$\leqslant 3$
105	7×10^1	$\leqslant 1 \times 10^2$	$\leqslant 2$

EMC virus injected intramuscularly can be shown to spread throughout the body. The following experiment suggests, however, that the virus can actually multiply in the calf muscle itself, in addition to multiplying elsewhere and merely spreading into the calf muscle secondarily.

Five-day-old mice from the DBA inbred strain were inoculated intramuscularly with 0·01 ml. of a 10^{-7} dilution of the standard EMC pool. Thirteen hours later, before any appreciable virus multiplication had occurred, the injected muscles were removed and transplanted into the right popliteal cavities of adult, relatively resistant, DBA mice. The dose used was too small to kill the adult mice in the absence of multiplication. To control the possibility that some multiplication had already taken place in the young mice, half the muscles were frozen and thawed twice ($-70 \rightleftharpoons +37°$ C.) before transplantation. This treatment kills the cells and thus prevents any further virus multiplication but does not destroy the virus. When the muscles were transplanted direct, seven out of eight host mice died; but when they were frozen first, six out of seven hosts survived. Fisher's exact test (Fisher, 1950; section 21.02) gives the probability of getting such a large departure from equality by chance alone as less than one in a hundred. This result suggests that virus multiplication was in fact taking place locally, in the injected muscle.

(*c*) *Haemagglutination tests*

The failure to demonstrate any increase in infectivity following the inoculation of small amounts of virus into the muscles of relatively resistant adult mice does not exclude the possibility that such muscles might be producing haemagglutinating, non-infective virus particles in large amounts.

In order to investigate this possibility, groups of 5-day-old and 30-day-old mice were given intramuscular injections of EMC virus (eight intracerebral LD_{50}'s per muscle) or the GDVII strain of mouse encephalomyelitis (thirty intracerebral LD_{50}'s per muscle). After 48 hr. for the EMC group, and 72 hr. for the GDVII group, homogenates of the injected muscles were tested by standard techniques for their ability to agglutinate sheep red cells (Gard & Heller, 1951) and human group O red cells (Lahelle & Horsfall, 1949), respectively.

Table 3. *Results of haemagglutination tests on tissues inoculated with EMC and GDVII virus*

	EMC Agglutination titre		GDVII Agglutination titre	
Tissue	Specific	Non-specific	Specific	Non-specific
5-day-old muscle, virus infected	1/80	1/20	1/160	1/20
5-day-old muscle, normal	⩽1/20	1/20	⩽1/20	1/20
30-day-old muscle, virus infected	⩽1/20	1/20	⩽1/20	1/20
30-day-old muscle, normal	⩽1/20	1/20	⩽1/20	1/20
Infected brain from stock suspension	1/1280	—	1/6400	—

The results of the tests are shown in Table 3. The 1/10 and 1/20 dilutions of all the muscle suspensions, including controls from untreated animals, showed a small amount of non-specific haemagglutination. On both tests specific haemagglutinating activity could be demonstrated in the virus-infected muscle suspensions from 5-day-old mice, but was absent in the muscle suspensions from 30-day-old mice. The haemagglutination tests are unfortunately very much less sensitive than infectivity tests, as can be seen from the tests on the stock virus suspensions. It is, however, clear that no large amounts of haemagglutinating non-infective virus particles are present in the older muscles.

Virus multiplication in adult mice

Although no increase of virus could be demonstrated in the muscles of adult mice following the injection of very small amounts of virus, it was possible that multiplication might occur if the virus dosage approached the level to which mice of that age were susceptible. In this case, however, the method employed above, of comparing the amount of virus in the muscle after 48 hr. with the amount of virus injected initially, may not detect virus multiplication: only a small fraction of the initial inoculum may be involved in the multiplication process, and the amount of virus extracted at the end of the experiment may therefore, in spite of multiplication, be no greater than that injected initially.

If, however, a number of relatively resistant mice are injected with a large

dose of virus, and the virus content of infected muscles estimated at varying intervals after inoculation, the later samples may be found to contain more virus than the earlier ones, even though both contain less than was originally injected.

Six-month-old mice were injected intramuscularly with a suspension of EMC or GDVII virus sufficiently concentrated to kill all the inoculated mice. Groups of mice were killed at intervals after inoculation and the virus content of their muscles was estimated.

Table 4. *Virus content of the muscles of older mice following inoculation of large amounts of EMC and GDVII virus*

Virus	Intracerebral LD_{50}'s per muscle in inoculum	Intracerebral LD_{50}'s present in muscle after 5 min.	5 hr.	6 hr.	24 hr.	48 hr.	96 hr.	192 hr.
EMC	7×10^3	1×10^2	—	—	—	2×10^3	—	1×10^2
	7×10^3	3×10^1	—	2×10^2	5×10^2	2×10^3	—	—
GDVII	1×10^3	5×10^1	8×10^1	—	2×10^3	1×10^3	0	0

The results of one such GDVII and two EMC experiments are given in Table 4. In all three experiments only a small fraction of the inoculated virus could be detected when the muscles were dissected out immediately after inoculation (see below). After the initial sharp decline, the amount of detectable virus in the inoculated muscle rose steadily up to 48 hr. for the EMC virus, but never reached the level that had been injected originally. The results of the two experiments on EMC virus agree closely. The GDVII virus reached a peak at 24 hr., at which time there was slightly more virus present in the muscle than had been injected initially.

Thus, it appears that inoculation of a dose of virus, large enough to produce infection, is followed by multiplication of the virus whether in adult or young animals; although in the former it may be impossible to demonstrate increase of virus content relative to the necessarily large inoculum. It is not possible to compare the extent of multiplication in young and adult animals as we do not know what proportion of the injected virus undergoes multiplication.

Apparent initial disappearance of virus from site of injection

Table 4 shows that when either EMC or GDVII virus is injected into a calf muscle and the muscle dissected out within 5 min. of injection, its virus content is only 0·5–5 % of that of the inoculum.

Cairns (1950) showed that only 2–8 % of intracerebrally inoculated bacteriophage was still present in the brain and meninges of mice 5 min. after inoculation. The rest of the inoculum was dispersed about the body. Total recovery of the phage was obtained from a suspension of a whole mouse made in a Waring blendor, indicating that the whole inoculum was still detectable. On the other hand, many observations suggest that once virus has been adsorbed by a susceptible cell and has begun its multiplication cycle it ceases to be infective.

To determine whether any part of the apparent initial disappearance of virus from the inoculation site was due to this latter phenomenon rather than to dispersal around the body, the percentage of injected virus detectable in the muscle immediately after inoculation was estimated in live and dead mice, freshly isolated muscles, and muscle suspensions. An inoculum containing 6×10^3 intracerebral LD_{50}'s of EMC virus was used and the virus content of all suspensions was estimated by intracerebral titration.

The results of the experiment are given in Table 5. There appears to be a considerable loss of infectivity of the virus inherent in the technique, since only about one-third of the infectivity was recovered whether the inoculum was dropped into a muscle suspension or into a corresponding volume of the standard virus diluent. When the virus was injected into an isolated calf muscle, less than 20 % was recovered, compared with 30–40 % from the muscle suspension. If significant, this difference may spring from some virus particles adsorbing onto the live cells in the muscle and losing their infectivity. The still lower recovery (5 %) from the muscle of intact dead mice is presumably due to passive draining away of the inoculum, while the further drop to 1 % recovery in living mice must represent that portion of the inoculum which is carried away from the inoculation site by the blood stream and lymph.

The major part of the observed initial disappearance of virus from the inoculation site appears, therefore, to be due to active or passive dispersal around the body, rather than to any loss of infectivity of the virus.

DISCUSSION

Sabin (1941) showed that there exist in the adult animal regional barriers to the invasion of the central nervous system by a number of neurotropic viruses. For different viruses these blocks occur at different sites and may develop at different ages, thus accounting for some of the very wide divergence encountered in mode of development with age of host resistance to infection with neurotropic viruses. It is of interest to inquire whether the results described in the present paper throw light on the causal basis of the development with age of resistance to infection with the viruses studied.

When a neurotropic virus such as the GD VII strain of mouse encephalomyelitis is inoculated into a muscle, it has various obstacles to overcome before it can enter the central nervous system causing paralysis and death of the host. Alteration with age of any of these obstacles may play a part in the development of host resistance. First, the virus must enter some cell in the muscle, though not necessarily a muscle fibre. Once inside a cell it may multiply. Rustigian & Pappenheimer (1949) report multiplication of GD VII and other neurotropic viruses in the calf muscles of 3-week-old mice, though the interpretation of their results is complicated by the large dose of virus and large inoculum (0·2 ml.) used. Evidence to be reported elsewhere indicates that, for EMC virus, the primary site of multiplication outside the central nervous system may be the supporting, non-neural elements of peripheral nerve. For convenience the term 'muscle cell'

Table 5. *Loss of infectivity immediately after inoculation with* 6×10^3 *intracerebral* LD_{50}*'s of EMC virus*

Virus injected into										
Calf muscles of live mice		Calf muscles of dead mice				Freshly isolated calf muscles		Muscle suspension			Spun muscle suspension	Normal diluent
		Just dead		18 hr. dead								
Expt. 1	Expt. 2	Expt. 1	Expt. 2			Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 3		
Intracerebral LD_{50}'s detected immediately after inoculation	1×10^2	3×10^1	6×10^2	1×10^2	2×10^2	$1{\cdot}0 \times 10^3$	$1{\cdot}1 \times 10^3$	$2{\cdot}3 \times 10^3$	$2{\cdot}7 \times 10^3$	$2{\cdot}0 \times 10^3$	$1{\cdot}8 \times 10^3$	$2{\cdot}3 \times 10^3$
% Infectivity lost	99		95			82		61			70	61

will be used to denote whatever non-neural cell in the muscle is involved in virus multiplication. Finally, the virus must pass from the muscle cell into the central nervous system. A certain concentration of virus may be needed before there is a high probability of a single infective dose entering the nervous system. Thus, when a small dose of virus is given, some local multiplication of virus may be required before it attains this critical concentration. Hence, there are at least three host properties alteration of which with age might be expected to affect resistance: (1) permeability of the non-neural muscle cell to virus; (2) ability of this cell to support virus multiplication; and (3) critical concentration of virus in the muscle cell which will permit entry into the central nervous system.

In the present paper we have demonstrated, for EMC and two strains of Coxsackie virus, that there exists an exact correlation between susceptibility to injection and virus multiplication in the muscle or brain of mice of the corresponding age providing that only a very small amount of virus is injected. MacDonald & Sanders (1959) find a similar correlation using GDVII virus. Wherever some degree of resistance to virus infection is encountered, no appreciable amount of multiplication of a small inoculum of virus can be demonstrated.

This correlation might suggest that the greater resistance of older mice to intramuscular inoculation is in some way a result of inability of the virus to multiply in the muscle.

If a small intramuscular dose of virus cannot invade the central nervous system without first undergoing some degree of multiplication, decrease in the ability of the muscle to support virus multiplication might lead to increased resistance in three ways. (*a*) The muscles of animals above a certain age might be unable to support virus multiplication at all. This is inconsistent with the finding that resistance to intramuscular inoculation may, as in the case of EMC virus, continue to increase throughout life. Also, we have shown above that virus multiplication *can* occur in older, relatively resistant animals if the initial dose is large. (*b*) The rate of virus multiplication might decrease with age. This by itself would only lead to increased resistance in the form of lengthened incubation periods: the mice would eventually succumb whatever their age. This is not what is observed. (*c*) The rate of virus multiplication might decrease with age and the immune response of the host might in the older animals intervene during the prolonged period of preliminary multiplication, neutralizing the virus before it had attained the critical level for entry into the central nervous system. If this were so, the intervals between inoculation and the appearance of symptoms of damage to the nervous system (incubation period) should not only lengthen as the virus dosage is decreased (this is a common finding), but the *slope* of incubation period plotted against virus dosage should decrease with age. In other words, the mean interval between the lengths of the incubation periods on adjacent tenfold dilutions should increase with the age of the host. Table 6 shows that there is little sign of any such effect with EMC virus, and none at all with GDVII virus (data on incubation periods taken from intramuscular titrations carried out in the course of the work described in this paper and in MacDonald and Sanders, 1959).

Increase with age of the critical concentration of virus in the muscle cell, which will permit entry into the central nervous system, would again lead to longer incubation periods in older mice, but would not save them from eventual invasion of the central nervous system. Nor would it affect the amount of virus multiplication occurring in the muscle.

All the data are, however, accounted for on the assumption that the accessibility or permeability of the muscle cells to virus decreases with age—that is, that the probability of a given virus particle finding and entering a susceptible cell is very much higher in young than in old mice. Unless it enters a cell, the virus can

Table 6. *Mean differences in the length of the incubation period in mice inoculated intramuscularly with adjacent tenfold dilutions of virus*

Virus	Age of mice (days)	Dilutions available for analysis	No. of differences between adjacent dilutions	Total no. of mice contributing to mean difference	Weighted mean of the difference in incubation period on adjacent dilutions
EMC	5	10^{-4}–10^{-8}	4	33	+0·45
	10	10^{-5}–10^{-7}	2	14	+0·37
	15	10^{-4}–10^{-8}	4	20	+0·39
	20–24	10^{-4}–10^{-7}	3	30	+0·94
	33	10^{-1}–10^{-3}	2	29	+0·56
	40–60	10^{-4}–10^{-7}	3	48	+0·68
	100–120	10^{-4}–10^{-6}	2	25	+1·01
GDVII	5	10^{-2}–10^{-4}	2	25	+0·60
	10	10^{-1}–10^{-5}	4	35	+2·10
	15	10^{-2}–10^{-4}	2	12	+0·85
	21–23	10^{-1}–10^{-5}	4	173	+1·50
	29–30	10^{-1}–10^{-3}	2	33	−0·33
	41	10^{-1}–10^{-4}	3	23	+1·70
	180	10^{-1}–10^{-4}	3	12	+0·66

In order to calculate the weighted means given in the last column, each difference between incubation periods on adjacent dilutions was weighted with the harmonic mean of the numbers of mice dying on the two dilutions concerned.

neither multiply nor invade the central nervous system; so that if, in older mice, a very large number of virus particles had to be injected into a muscle to ensure that any virus actually got inside the cells, this would account both for the observed increased resistance to intramuscular inoculation which develops with age, and also for the observations on virus multiplication described above. Failure to detect any evidence of virus multiplication in the muscles of older mice would then be due, not necessarily to any decrease in ability of virus to multiply in an older muscle cell, but rather to inability of the virus to enter the muscle cell at all. If we inject a large dose of virus into the muscle of an older mouse, the actual amount of virus which is able to multiply will obviously be small if there is only a small probability of a given virus particle entering a muscle cell; so that even if it multiplies at the same rate as in young mouse muscle, the amount of virus which we can extract at the end of our experiment may be no greater than the large

virus dose which we injected initially. This is exactly the picture which we obtained when virus multiplication was studied in the muscles of 'resistant' mice following a large dose of EMC or GD VII virus.

Additional support for the theory that decreased accessibility of the cells to virus plays a part in the development of age resistance is provided by the finding that three injections at daily intervals of 12 % physostigmine sulphate, a substance which is known to increase the permeability of several types of cell (for references see Greig & Mayberry, 1951), increased nearly tenfold the susceptibility of relatively resistant mice to GD VII virus when both were injected into the same muscle. The effect is significant at the 1 % level. Injection of distilled water produced no increase in susceptibility.

Preliminary results with EMC and GD VII viruses indicate that adult muscle which is growing rapidly as a result of recent re-innervation, also resembles young muscle in its hospitability to virus multiplication; while Rowe (1953) finds that type A Coxsackie viruses will grow in denervated adult mouse muscle, which shows some biochemical similarities to young muscle.

The four viruses used in the work described in this paper differ in their mode of invasion of the host organism and in their tissue affinities. Moreover, the work on EMC virus shows that the development with age of resistance to infection may vary in rate and extent according to the particular peripheral route by which the virus is administered. It is clear that no single hypothesis can account for all the age resistance phenomena observed. Our results suggest, however, that changes with age in the accessibility of cells to virus may be particularly relevant in certain cases.

SUMMARY

1. The susceptibility of mice of different ages to intramuscular, intraperitoneal, and oral administration of EMC virus, and to intracerebral and intramuscular injection of both the Texas and the Connecticut-5 strains of Coxsackie virus have been studied. In all cases susceptibility decreases with age. The development of resistance to a given virus may vary in rate and extent according to the route by which the virus is given.

2. The multiplication of small amounts of EMC virus in muscle, and of the Texas and Connecticut-5 strains of Coxsackie virus in both muscle and brain, has been studied in mice of different ages. A very high degree of multiplication of all three viruses was observed in both muscle and brain of young mice, but no significant multiplication of the EMC virus in older muscle could be detected with these small inocula, nor of either of the Coxsackie strains in older muscle or brain.

3. Multiplication of EMC virus in young mice was shown to take place within the muscle itself. Haemagglutination tests showed that less haemagglutinin, as well as less infective virus, was produced in the muscles of older mice following the injection of small amounts of virus.

4. Multiplication of EMC and GD VII viruses could be demonstrated even in older mice if a sufficiently large amount of virus was inoculated initially.

5. The rapid initial disappearance of infectivity from the site of inoculation was shown to be mainly due to dispersal of inoculated virus around the body.

6. The simplest hypothesis which will accommodate the observed data on virus multiplication and on the development of resistance with age appears to be that the accessibility of the host cell to virus decreases with age. We found no evidence that the ability of the host cell to support virus multiplication declines with age.

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