THE EFFECT OF VACCINES AND OTHER SUBSTANCES UPON THE COURSE OF NEUROTROPIC VIRUS INFECTION

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(With 2 Figures in the Text)

It is now well established that intramuscular inoculation with combined diphtheria–pertussis prophylactics can affect the course of poliomyelitic infection in children. Localization of paralysis in the limb injected with vaccines was reported by McCloskey (1950), Martin (1950), Geffen (1950), and Hill & Knowelden (1950). Hill & Knowelden (1950) and Benjamin (1950) showed conclusively that such inoculations can also increase the incidence of overt paralytic symptoms. Subsequent confirmatory evidence has been collected by Grant (1953) and other workers. Inoculations preceding the onset of symptoms by more than a month appear to have no effect upon either the localization or incidence of paralysis.

Other factors which have been shown clinically to modify the distribution and incidence of paralysis during poliomyelitis include tonsillectomy (for references, see Medical Research Council Committee, 1955; Anderson & Rondeau, 1954), exercise (Russell, 1947, 1949), and the injection of penicillin (Banks, 1954) and other substances (Schwartzman, 1950; Rosen & Thooris, 1953; Townsend-Coles & Findlay, 1953).

Some experimental studies have been made of the effects of vaccine injections upon the course of infection with neurotropic viruses. Findlay & Howard (1950) found that intravenous injections of diphtheria toxoid and pertussis vaccines into mice inoculated into the brain with poliomyelitis virus (Lansing strain), shortened the incubation period of the infection; Milzer, Weiss & Vanderboom (1951) claim similar results with intraperitoneal inoculation of vaccines, but publish no figures; Dean, Cohen & Dalldorf (1951) found that if diphtheria toxoid or pertussis vaccine was injected into the limb muscle of a mouse before or at the same time as an intracerebral injection of mouse encephalomyelitis virus (TO strain), paralysis tended to be localized in the injected limb. More recently Bodian (1954) has made an analytical study of the effect of intramuscular inoculation of vaccines and other substances into monkeys inoculated into a limb muscle or into the heart with poliomyelitis virus. He observed an increase in the incidence of paralysis with marked localization in the injected limb. His evidence as to the mechanism of the provoking and localizing effect of intramuscular inoculation is, however, at variance with that obtained by Verlinde, Kret & Wyler (1955) in a similar study.

The present work is concerned with the effect of vaccine injections in mice both upon the susceptibility to overt virus infection and upon the localization of paralysis. Since peripheral inoculation of virus achieves a closer parallel with the clinical picture than intracerebral inoculation, two viruses have been

used which, unlike the TO strain of encephalomyelitis or the Lansing strain of poliomyelitis, will produce infection when inoculated peripherally into adult mice. The viruses of both encephalomyocarditis (EMC) and the GDVII strain of encephalomyelitis (GDVII) when injected into the limb muscles of mice produce a flaccid paralysis similar to that of human poliomyelitis. Neither virus is, however, immunologically related to the viruses of poliomyelitis and this should be borne in mind when assessing the clinical relevance of the experimental results described below.

MATERIALS AND METHODS

Viruses

The viruses of encephalomyocarditis (EMC) and the GDVII strain of encephalomyelitis (GDVII) were kindly provided by Dr F. K. Sanders in the form of freshly prepared mouse-brain suspensions.

Standard pools of GDVII and EMC viruses were made up by homogenizing infected mouse brains in a chilled Waring Blendor for 5 min. Distilled water was used as diluent for the GDVII virus and 10% normal rabbit serum in isotonic phosphate-buffered saline (pH 7·2) for the EMC virus. The EMC virus suspension was centrifuged at 2750 r.p.m. for 15 min. before use, and the supernate decanted and diluted as required. The virus pools were stored at $-70°$ C., and did not alter significantly in infectivity over the period during which the experiments described were carried out. The 50% lethal dilutions of the GDVII and EMC pools (determined by injecting serial tenfold dilutions intracerebrally into groups of eight mice) were $10^{-8·0}$ and $10^{-8·4}$ respectively.

Vaccines

The vaccines were obtained from Dr R. L. Vollum of the Public Health Laboratory, Oxford, and were stored at $+3°$ C. They consisted of Pertussis Vaccine (AIII, specially manufactured for the Medical Research Council) and Diphtheria Prophylactic, alum-precipitated toxoid 'Wellcome' (APT). They were used diluted 1:2 with distilled water, and where a mixture was used, this was prepared with one part of pertussis vaccine, one part of APT, and four of distilled water. Both prophylactics were injected intramuscularly or intracerebrally on many occasions, and in the absence of virus were never observed to have any paralytic effect.

Mice

Male mice from a commercial dealer were used, 3–4 weeks of age. For any one experiment, all mice were from a single batch of the same age, and were distributed at random among the various treatments and dosages. The mice were fed *ad lib.* with a standard pellet diet (Rowett no. 86), and kept at a temperature of $70° \pm 2°$ F.

Inoculations

Intramuscular inoculations were made into the calf muscles of the hind leg, intravenous inoculations were made into a tail vein. For intramuscular and

intracerebral inoculations a standard volume of 0·03 ml. was given, using a 0·25 in. 27-gauge needle. For intravenous and intraperitoneal inoculations the volumes given were 0·1 and 0·3 ml. respectively, using a 0·75 in. 19-gauge needle.

Calculation of 50 % *lethal dilution*

LD_{50}'s were calculated either by the method of Reed & Muench (1938) or by the method of probit analysis, as described by Finney (1947). The second method was used where it was desired to obtain estimates of an improved and known level of precision. It enables one to demonstrate a significant difference in susceptibility (produced, for example, by some experimental treatment) from smaller numbers of animals than the cruder method would require. Differences in susceptibility between groups were estimated in terms of the mean probit difference, or vertical distance between the two parallel probit lines (see Finney, 1947).

RESULTS

A. *Virus injected into the brain*

In two experiments, an intracerebral injection of a serial dilution of GDVII virus was followed after 24 or 48 hr. by an intramuscular injection of mixed pertussis and diphtheria prophylactic. No effect was observed on mortality, incubation period, or distribution of symptoms.

In a third experiment, the mixed prophylactic was injected after a 24 or 48 hr. interval into the brain. Again, there was no effect on mortality or type of symptoms, but there was a suggestion that the symptoms were appearing earlier in the vaccine-treated than in the control mice.

Table 1. *Effect of intracerebral injection of vaccines on the incubation period following intracerebral injection of a* 10^{-7} *dilution of GDVII virus*

Day after inoculation of virus on which first symptoms appeared	No. of mice		No. of experimentals expressed as percentage of total number of mice (%)
	Controls	Experimentals	
3rd day	4	15*	79
4th day	17	10	37
5th day	9	3	25
Totals	30	28	—

* One of these fifteen mice showed symptoms late on the 2nd day, but has been included here for convenience.

A further batch of mice was, therefore, given intracerebral injections of a 10^{-7} dilution of GDVII virus, and this was followed in half the batch by an intracerebral injection of the mixed prophylactic 24 hr. later. The results from this experiment and the previous one have been combined in Table 1, which gives the number of mice first showing symptoms during each 24 hr. period after inoculation. The proportion of those mice first showing symptoms, which is found in the experi-

mental rather than in the control group, decreases significantly on successive days ($\chi^2_{(1)} = 9{\cdot}40$, $P < 0{\cdot}01$; see Holt, 1948), so that the treatment does appear to shorten the incubation period. The length of the incubation period proved also to be significantly more variable in the experimental group ($z = 0{\cdot}3364$, $P < 0{\cdot}05$). This would be expected if the mice varied in their response to the vaccine as well as in their response to the virus.

Thus, when GDVII virus was introduced directly into the central nervous system (C.N.S.) subsequent peripheral injection of vaccines had no influence upon the course of the infection; while intracerebral injection of vaccines accelerated the onset of symptoms of cerebral damage.

B. *Virus injected peripherally*

The course of infection after intramuscular inoculation of EMC and GDVII viruses

Extensive data on the distribution of first symptoms after intramuscular inoculation were abstracted from previous work on GDVII and EMC viruses (Sanders, McLaren & MacDonald, unpublished). In Table 2, the first symptoms are tabulated as flaccid paralysis of one or other of the four limbs, or as symptoms referable to brain damage (circling, convulsions, spastic paralysis, prostration). Mice which, when first observed to be ill, were paralysed in more than one limb, or which

Table 2. *Distribution of first symptoms after inoculation of GDVII and EMC viruses into the right hind leg*

	No. of mice showing				
		Flaccid paralysis of			
		Front limb		Hind limb	
Virus	Cerebral symptoms	Right	Left	Right	Left
GDVII	2	4	7	83	1
EMC	49	11	5	39	6

showed both cerebral and paralytic symptoms, have been omitted. With EMC virus, there is a much greater tendency to produce cerebral symptoms than with GDVII virus. In both, there is a strong localization of paralysis in the injected limb as compared with the opposite hind limb; this is particularly striking in the case of GDVII virus.

When GDVII virus is injected into a calf muscle it is believed to reach the C.N.S. by travelling along the sciatic nerve, since interruption of this nerve, within a certain time interval after inoculation, protects mice from infection (Sanders, 1953). This may account for the marked localization of paralysis observed in the injected limb, as the virus will tend to damage first those cells nearest its point of entry into the nervous system. In order to investigate the route of entry of EMC virus into the C.N.S., serial dilutions were injected into the calf muscle of two groups of mice. In one group, the sciatic nerve on the injected side had been cut at the level of the top of the femur on the previous day, a procedure known to protect mice similarly

inoculated with GDVII virus. The results are shown in Table 3. The absence of any protective effect of sciatic nerve section suggests that EMC virus reaches the C.N.S. by a different route, probably by way of the blood circulation, since it is known to be present in high concentration in the blood shortly after intramuscular inoculation (Strahan, personal communication).

Table 3. *Effect of sciatic nerve section upon susceptibility to EMC virus inoculated intramuscularly*

Sciatic nerve	Dilution of standard virus pool				LD_{50} (Reed & Muench, 1938)
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
Intact	5/5	5/5	4/5	2/6	$10^{-6\cdot6}$
Cut	5/5	5/5	4/5	1/6	$10^{-6\cdot5}$

Denominator represents number of mice injected; numerator represents number which died.

That GDVII virus also is present in the blood after injection into the calf muscle was shown by demonstrating the infectivity of samples of blood removed by cardiac puncture at intervals after the injection of virus. Small amounts of GDVII virus were detected in the blood at each interval tested (10 min., 7, 24 and 48 hr.).

Nature of localizing stimulus in intramuscular inoculation

In view of the clinical findings, an attempt was made to determine whether the localization of paralysis in a limb inoculated with virus (Table 2) was an effect of the virus itself in the limb, or was a non-specific effect of intramuscular inoculation.

In the first experiment, groups of mice received intramuscular inoculations o GDVII or EMC virus, half into the right hind leg and half into the left. Both here and in the next experiment there was no stronger tendency for localization onef side than the other, and the results for the two halves are therefore combined. The opposite hind leg was either left untreated, inoculated with mixed diphtheria–pertussis prophylactic, or inoculated with a suspension of normal mouse brain prepared in the way already described for the standard EMC virus pool. Normal mouse brain suspension alone produced no ill effects when injected into mice.

The results are shown in Table 4. With GDVII virus, even in the groups where both hind limbs had received injections, the tendency for localization of paralysis in the limb injected with virus was complete and highly significant ($P < 0{\cdot}001$). With EMC virus, on the other hand, of the eight mice showing hind-limb paralysis as a first symptom, four were affected in the limb inoculated with virus and four in the limb inoculated with normal mouse-brain suspension. In spite of the small numbers, this just differs significantly at the 5% level (using Yates's correction $\chi^2_{(1)} = 3{\cdot}81$) from the distribution of hind-limb paralysis in the control data.

In the second experiment, the GDVII and EMC viruses were injected by the intravenous and intraperitoneal routes respectively, so that the resulting paralytic symptoms would be expected to affect both limbs equally. In the GDVII series two injections of either the mixed prophylactic or the normal mouse-brain suspension were given into the calf muscle of one or other hind leg, one 72 hr. before the

Table 4. *Distribution of first symptoms after intramuscular inoculation of EMC and GDVII viruses into one leg, and vaccines or normal mouse brain into the other leg. (Control data for both viruses taken from Table 2)*

		No. of mice showing					
			Flaccid paralysis of				
			Front limb		Hind limb		
Virus	Substance injected into opposite leg	Cerebral symptoms	Side injected with virus	Opposite side	Side injected with virus	Opposite side	Total injected*
EMC	Normal mouse brain	11	5	5	4	4	40
	Control	49	11	5	39	6	—
GDVII	Normal mouse brain	1	0	0	4	0	8
	Vaccine mixture	0	0	0	6	0	8
	Control	2	4	7	83	1	—

* In this and in the succeeding table, the total number of mice injected in each group usually exceeds the number classified for first symptoms. The remaining injected mice either (1) survived for the duration of the experiment without showing symptoms, or (2) died without any preliminary symptoms having been observed, or (3) showed more than one type of symptom when first observed to be ill.

inoculation of the virus and the other immediately after. In the EMC series a single injection of the mixed prophylactic or the normal mouse-brain suspension was given into the muscles of one or other hind leg immediately after the inoculation of the virus. Control groups in each series received virus only.

The results are shown in Table 5. With EMC virus the vaccine injections exerted no localizing effect: both in the control group and in the vaccine group the small number of hind-limb paralyses observed were distributed equally between the two sides. But of the mice injected with normal mouse brain, seventeen showed paralysis of the inoculated limb and only two of the opposite hind limb. This represents a highly significant departure from equality ($\chi^2_{(1)} = 11{\cdot}9$, $P < 0{\cdot}001$), and indicates a localizing effect at least as strong as is observed following intramuscular inoculation with EMC infected mouse brain (Table 2). With GDVII virus, comparing the proportion of affected mice showing paralysis of the left hind leg in the three groups, both the group treated with normal mouse brain and the group treated with vaccines differ significantly from the controls by Fisher's (1925–50) exact method ($P < 0{\cdot}01$ and $P < 0{\cdot}02$ respectively). When comparing the efficacy of the mixed vaccine injections as a localizing agent in the EMC and GDVII series, it should be remembered that the mice in the GDVII series received an additional injection of vaccines 72 hr. before the virus was given.

To sum up, when GDVII virus is inoculated into a limb muscle the subsequent localization of paralysis is unaffected by the injection of non-viral substances into the opposite limb; the paralysis presumably results from the injected virus reaching

the C.N.S. by way of the sciatic nerve. In contrast to this, the paralysis following the injection of EMC virus into a limb muscle appears to be due to the local effect of the inoculation itself, since the injection of normal mouse brain suspension into the opposite limb results in a shift in the distribution of symptoms. A localization just as extreme is seen when non-viral substances are injected into the limb muscles of mice previously inoculated intraperitoneally with EMC virus, or intravenously with GDVII virus.

Table 5. *Distribution of first symptoms after intraperitoneal inoculation of EMC virus or intravenous inoculation of GDVII virus, and intramuscular inoculation of vaccines or normal mouse brain*

		No. of mice showing					
			Flaccid paralysis of				
			Front limb		Hind limb		
Virus	Substance injected into calf muscle	Cerebral symptoms	Treated side	Opposite side	Treated side	Opposite side	Total injected
EMC	Normal mouse brain	34	9	7	17	2	80
	Vaccine mixture	21	5	8	3	3	40
GDVII	Normal mouse brain	2	0	1	4	1	10
	Vaccine mixture	7	1	2	6	1	20
			Left	Right	Left	Right	
EMC	Control	9	2	1	2	2	20
GDVII	Control	9	3	1	0	1	20

Effect of intramuscular vaccine injections upon susceptibility

Mixed pertussis and diphtheria prophylactic was injected intramuscularly at different intervals before and after the inoculation of serial dilutions of GDVII virus into the same muscles. The results are shown in Table 6. The difference between controls and treated was very slight when the vaccine was injected 48 hr. before

Table 6. *Effect of intramuscular injection of vaccines at different time intervals before and after intramuscular injection of GDVII virus (Expt. 1)*

	Dilution of standard GDVII pool					
Treatment	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Log LD_{50} (probit method)	Mean probit difference between controls and treated
Control	2/5	1/5	1/5	0/5	−2·07	—
Vaccine 48 hr. before virus	2/5	2/5	1/6	0/4	−2·34	−0·19 ± 0·54
Vaccine 24 hr. before virus	3/5	0/5	2/5	0/5	−2·42	−0·25 ± 0·54
Vaccine immediately after virus	3/5	1/5	2/5	0/5	−2·64	−0·41 ± 0·54
Vaccine 24 hr. after virus	4/5	4/5	0/5	0/5	−2·94	−0·63 ± 0·56
Vaccine 48 hr. after virus	5/5	5/5	0/5	0/5	−3·50	−1·03 ± 0·57

Denominator represents number of mice injected; numerator represents number which died. Calculated slope of probit line = 0·72 ± 0·17.

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the virus, but increased steadily the later the vaccine was given. When the vaccine was given 48 hr. after the virus, the treated mice were about thirty times more susceptible than the controls. All five differences are in the direction of increased susceptibility, and in aggregate the effect is statistically significant. The relation between increase of susceptibility in the treated mice and time of inoculation is clearly significant, as can be seen from Fig. 1, and is consistent with the clinical finding that vaccine injections exert their greatest influence upon susceptibility to

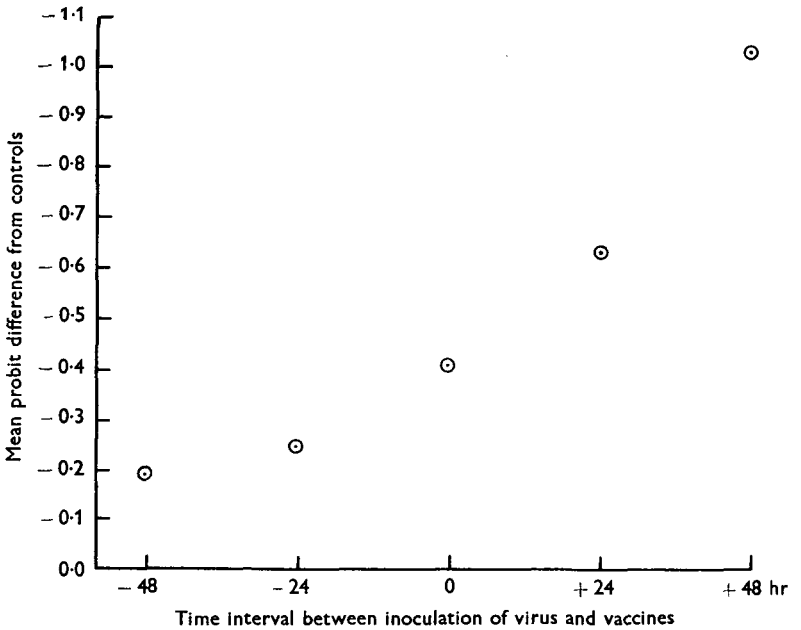

Fig. 1. Effect of intramuscular inoculation of vaccines upon susceptibility to intramuscular inoculation of GDVII virus. (From Table 6.)

poliomyelitis if given while the patient is incubating the disease or in the early non-paralytic stage, and have no effect if given more than four weeks preceding the onset of illness. It agrees also with Bodian's (1954) observation that the provoking effect of intramuscular injections in monkeys decreased sharply if the injections were given more than two weeks before the poliomyelitis virus was inoculated.

The experiment described above was carried out in February 1951. Six similar experiments were undertaken during the following year. Two batches of vaccine were used. The first was obtained in February 1951; the diphtheria toxoid had been prepared in October 1950, but the date of manufacture of the pertussis vaccine was not known. The second batch was obtained in December 1951; the diphtheria toxoid was from a new lot prepared in October 1951, while the pertussis vaccine was from the same lot as previously. The results of all seven experiments are summarized in Table 7.

The effect of the vaccine injections upon susceptibility to virus appeared to alter during the course of the year. A preliminary account of this phenomenon has already been published (McLaren, 1953). As described above, in the first experi-

ment (February 1951) the vaccine treatment increased the susceptibility of the mice to virus at all five time intervals tested ($P < 0{\cdot}05$). A month later, a very slight, non-significant increase in susceptibility was obtained. In September, injection of the same vaccine mixture 48 hr. after the injection of virus was found to *decrease* nearly tenfold the susceptibility of the mice to virus—i.e. to increase their resistance. Larger batches of mice were used in this experiment, and the result is significant at the 5% level. In a further experiment in November, an increase of resistance was again obtained, whether the vaccine was injected 48 hr.

Table 7. *Alteration with time in effect of intramuscular injection of vaccines upon susceptibility to GDVII virus*

Expt.	Month	Batch of vaccines	Interval between virus and vaccine injections (hr.)	Mean probit difference from controls	Joint estimate of mean probit difference
1	Feb. 1951	1	−48	$-0{\cdot}19 \pm 0{\cdot}54$	$-0{\cdot}49 \pm 0{\cdot}24$ ($P < 0{\cdot}05$)
			−24	$-0{\cdot}25 \pm 0{\cdot}54$	
			+0	$-0{\cdot}41 \pm 0{\cdot}54$	
			+24	$-0{\cdot}63 \pm 0{\cdot}56$	
			+48	$-1{\cdot}03 \pm 0{\cdot}57$	
2	Mar. 1951	1	+48	$-0{\cdot}16 \pm 0{\cdot}51$	$-0{\cdot}17 \pm 0{\cdot}36$
			+96	$-0{\cdot}18 \pm 0{\cdot}51$	
3	Sept. 1951	1	+48	$+0{\cdot}79 \pm 0{\cdot}38$	$+0{\cdot}80 \pm 0{\cdot}24$ ($P < 0{\cdot}001$)
4	Nov. 1951	1	−48	$+1{\cdot}00 \pm 0{\cdot}57$	
			+48	$+0{\cdot}74 \pm 0{\cdot}54$	
5	Dec. 1951	1	+48	$+0{\cdot}70 \pm 0{\cdot}52$	
		2	+48	$-0{\cdot}19 \pm 0{\cdot}57$	$-0{\cdot}19 \pm 0{\cdot}57$
6	Feb. 1952	2	+48	$+0{\cdot}48 \pm 0{\cdot}46$	$+0{\cdot}48 \pm 0{\cdot}46$
7	Mar. 1952	2	+48	$+1{\cdot}36 \pm 0{\cdot}60$	$+1{\cdot}10 \pm 0{\cdot}32$ ($P < 0{\cdot}001$)
			+72	$+0{\cdot}89 \pm 0{\cdot}53$	
			+96	$+1{\cdot}12 \pm 0{\cdot}56$	

before or 48 hr. after the virus. In December, the second batch of vaccines was obtained, and both batches were tested simultaneously. The first batch again increased the resistance of the mice; the second batch, on the other hand, produced a small, non-significant increase in susceptibility. When tested again in February, the second batch of vaccines slightly decreased susceptibility, and in March decreased susceptibility markedly when injected either 48, 72 or 96 hr. after the injection of virus. On this last experiment, the joint estimate of the mean probit difference between treated and controls is $+1{\cdot}10 \pm 0{\cdot}32$. Expts. 3–5 on the first batch of vaccines give a joint estimate of $+0{\cdot}80 \pm 0{\cdot}24$. Either of these differences are of a magnitude which would be expected to occur by chance alone less than one in a thousand times; so there can be no doubt that the increase of resistance of the treated animals, although unexpected, is a real effect.

In Fig. 2 the mean probit differences between the control mice and those receiving vaccine 48 hr. after the injection of virus are plotted against time. Both batches of

vaccine show a clear downward trend. The time relations make it unlikely that the effect was due to a seasonal fluctuation; and as far as could be ascertained, no variations occurred in the experimental procedures. One must therefore assume that the properties of one or both of the vaccines was altering with age.

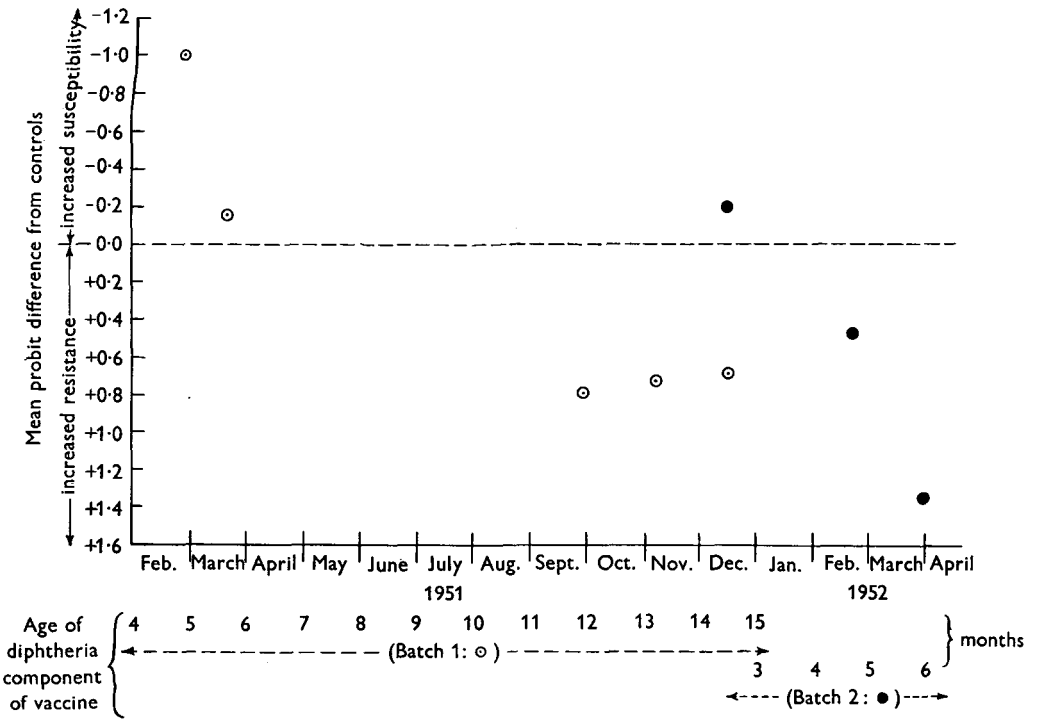

Fig. 2. Effect of vaccines of different ages upon susceptibility to GDVII virus (both injected into the same muscle, vaccines 48 hr. after virus). (From Table 7.)

Effect of intracerebral vaccine injections

Preliminary experiments suggested that the susceptibility of mice to intramuscular inoculation with GDVII virus is increased by the injection of mixed diphtheria and pertussis prophylactic into the brain either immediately or 48 hr. later. Two further experiments were undertaken to confirm this effect and to investigate its specificity.

In the first experiment, intramuscular inoculation of serial virus dilutions was followed immediately either by 'needling' (i.e. a hypodermic needle merely inserted into the brain as for inoculation) or by intracerebral inoculation of 2% sterile starch solution in distilled water, or of pertussis and diphtheria prophylactics, separately or mixed. Table 8 shows the mortalities with each virus dilution for the various treatments. The starch and 'needling' treatments clearly have no effect upon mortality, and these groups have been combined with the controls to give a joint estimate for log LD_{50} of $-3{\cdot}04 \pm 0{\cdot}29$. The combined estimate for log LD_{50} of the three vaccine-treated groups is $-3{\cdot}88 \pm 0{\cdot}41$, representing a sevenfold increase in susceptibility over the controls. The mean probit difference between

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Table 8. *Effect of intracerebral inoculation immediately after intramuscular injection of GDVII virus* (3-*week-old mice*)

Treatment	Dilution of standard GDVII pool 10^{-2}	10^{-3}	10^{-4}	Log LD_{50} (probit method)	Joint estimate of log LD_{50}
Control	4/5	2/5	2/5	$-3{\cdot}18 \pm 0{\cdot}50$	
'Needling'	5/5	2/5	0/5	$-2{\cdot}87 \pm 0{\cdot}49$	$-3{\cdot}04 \pm 0{\cdot}29$
2 % starch	4/5	1/4	2/5	$-3{\cdot}10 \pm 0{\cdot}53$	
Diphtheria toxoid	5/5	3/5	3/5	$-4{\cdot}00 \pm 0{\cdot}78$	
Pertussis vaccine	5/5	2/5	3/5	$-3{\cdot}73 \pm 0{\cdot}60$	$-3{\cdot}88 \pm 0{\cdot}41$
Mixed vaccine	5/5	3/5	3/5	$-4{\cdot}00 \pm 0{\cdot}78$	

Denominator represents number of mice injected; numerator represents number which died. Calculated slope of probit line $= 0{\cdot}75 \pm 0{\cdot}21$.

the two groups is $-0{\cdot}65 \pm 0{\cdot}26$, which is significant at the 5 % level. There is thus an indication that the injection of vaccines into the brain immediately after the intramuscular injection of virus slightly increases the susceptibility of the mice.

In the second experiment, intramuscular inoculation of serial virus dilutions was followed 48 hr. later by intracerebral inoculation of one of the following substances: distilled water, 0·90 % NaCl, 5·85 % NaCl (1·0 M), 0·32 % sodium citrate, normal rabbit serum, rabbit serum hyperimmune against GDVII virus, rabbit serum hyperimmune against EMC virus, sheep aqueous humour, mixed pertussis and diphtheria prophylactic. The injections of sodium citrate resulted in immediate convulsions and death in about a third of the mice; these have been omitted from subsequent

Table 9. *Effect of intracerebral inoculation* 48 *hr. after intramuscular inoculation of GDVII virus* (4-*week-old mice*)

Treatment	Dilution of standard GDVII pool 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Log LD_{50} (probit method)	Joint estimate of log LD_{50}
Control	9/10	5/10	0/10	—	—	$-1{\cdot}86 \pm 0{\cdot}34$	$-1{\cdot}86 \pm 0{\cdot}34$
5·85 % NaCl	3/5	4/5	2/5	—	—	$-2{\cdot}28 \pm 0{\cdot}46$	
0·32 % sodium citrate	3/4	4/4	1/6	—	—	$-2{\cdot}39 \pm 0{\cdot}47$	
0·9 % NaCl	5/5	4/5	1/5	—	—	$-2{\cdot}68 \pm 0{\cdot}54$	
Distilled water	5/5	5/5	0/5	—	—	$-2{\cdot}68 \pm 0{\cdot}54$	$-2{\cdot}79 \pm 0{\cdot}17$
EMC immune serum	4/5	4/5	2/5	2/5	—	$-2{\cdot}94 \pm 0{\cdot}43$	
Aqueous humour	4/5	4/5	4/5	1/5	—	$-3{\cdot}07 \pm 0{\cdot}44$	
GDVII immune serum	5/5	4/5	3/5	1/5	—	$-3{\cdot}18 \pm 0{\cdot}45$	
Normal rabbit serum	5/5	5/5	2/5	—	—	$-3{\cdot}29 \pm 0{\cdot}80$	
Mixed vaccine	5/5	4/5	5/5	5/5	0/5	$-4{\cdot}24 \pm 0{\cdot}45$	$-4{\cdot}24 \pm 0{\cdot}45$

Denominator represents number of mice injected; numerator represents number which died. Calculated slope of probit line $= 0{\cdot}82 \pm 0{\cdot}14$.

calculations, and sufficient additional mice used to allow for the inoculation mortality. In the absence of virus none of the other substances injected produced any harmful effects. Table 9 shows the mortalities on each dilution for the various treatments, together with the LD_{50}'s calculated by probit methods.

The vaccine injections exert a larger effect when injected 48 hr. after the virus than immediately after, increasing the susceptibility of the mice 300 times as compared with the controls. This increase is clearly significant: the mean probit difference from the controls is $-1{\cdot}95 \pm 0{\cdot}73$ ($P < 0{\cdot}01$). The other eight substances listed in Table 9 also exert some effect, giving a joint estimate for log LD_{50} of $-2{\cdot}79 \pm 0{\cdot}17$, which differs significantly both from the controls ($P < 0{\cdot}02$) and from the vaccine-treated mice ($P < 0{\cdot}01$). There is some indication that substances containing protein or protein derivatives produce a larger effect than the other substances tested.

When intravenous injections of GDVII virus are followed immediately by inoculation of mixed pertussis and diphtheria prophylactic into the brain, a marked increase in susceptibility is again observed (Table 10).

Table 10. *Effect of intracerebral injection of vaccines immediately after intravenous injection of GDVII virus*

	Dilution of standard GDVII pool			Log LD_{50} (probit	Mean probit
Treatment	10^{-2}	10^{-3}	10^{-4}	method)	difference
Control	4/5	1/5	0/5	$-2{\cdot}24$	$-1{\cdot}35 \pm 0{\cdot}49$
Mixed vaccines	4/5	4/5	4/5	$-4{\cdot}43$	($P < 0{\cdot}01$)

Denominator represents number of mice injected; numerator represents number which died. Calculated slope of probit line $= 0{\cdot}62 \pm 0{\cdot}31$.

GDVII virus has been shown above to be present in low concentration in the blood even after intramuscular inoculation. This concentration may be too low to permit direct entry of virus from the blood into the C.N.S. in the intact animal, but may suffice once the 'blood-brain barrier' is weakened by intracerebral inoculation (Macklin & Macklin, 1920). If this is the explanation of the facilitating effect which intracerebral vaccine injections exert, such injections should also abolish the protective influence which interruption of the sciatic nerve normally affords against GDVII virus inoculated into the calf muscle. A preliminary experiment suggested that this was indeed the case, but the results failed to reach formal significance ($P = 1/13$), and a second experiment was therefore undertaken.

Serial dilutions of GDVII virus were injected into the calf muscles of mice, half of which had had the sciatic nerve supplying the injected leg cut at the level of the head of the femur a few hours previously. For each dilution, the operated and intact mice were divided into three groups, one receiving no further treatment, the other two receiving intracerebral injections of mixed pertussis and diphtheria prophylactic (*a*) immediately after, and (*b*) 48 hr. after the virus injections. The results are given in Table 11. In the control group, section of the sciatic nerve afforded almost complete protection. It is characteristic that the only death occurred with the lowest dilution, where the concentration in the blood is perhaps high enough to permit passage through the 'blood-brain barrier' even when this is undamaged. In the two groups of mice injected intracerebrally with vaccines, on the other hand, section of the sciatic nerve gave little or no protection.

Table 11. *Effect of intracerebral injection of vaccines after intramuscular injection of GDVII virus into mice with sciatic nerves interrupted (4-week-old mice)*

Treatment	Sciatic nerve	Dilution of standard GDVII pool 10^{-1}	10^{-2}	10^{-3}	LD_{50} (Reed & Muench)
Control	Intact	4/5	2/5	0/5	$10^{-1\cdot7}$
	Cut	1/5	0/5	0/5	$< 10^{-0\cdot6}$
Vaccine immediately	Intact	4/5	3/5	1/5	$10^{-2\cdot2}$
	Cut	4/4	2/5	1/5	$10^{-2\cdot0}$
Vaccine at 48 hr.	Intact	4/5	4/5	2/5	$10^{-2\cdot5}$
	Cut	3/5	3/5	1/5	$10^{-2\cdot0}$

Denominator represents number of mice injected; numerator represents number which died.

Table 12. *Distribution of first symptoms following intramuscular injection of GDVII virus and intracerebral injection of vaccines*

	No. of mice showing symptoms of type	
	Paralytic	Cerebral
Controls (no vaccine)	23	0
Vaccines injected 0 hr. after virus	13	23
Vaccines injected 48 hr. after virus	19	1

Intracerebral injection of vaccines into mice inoculated intramuscularly with GDVII virus also affects the distribution of symptoms. Table 12 shows the symptoms observed when mixed vaccine was injected into the brain either immediately or 48 hr. after the intramuscular injection of GDVII virus. The mortality data for these mice are given in Tables 8 and 9, and have already been discussed. The mice are divided into those showing cerebral symptoms and those with flaccid paralysis of one or both hind limbs when first observed to be ill. None of the mice in the control group showed cerebral symptoms (see also Table 2). Where vaccine was injected into the brain immediately after the virus, on the other hand, twenty-three showed cerebral symptoms and only thirteen flaccid paralysis. This provides further confirmation that the intracerebral vaccine injections allow virus, given intramuscularly, to pass directly from the blood into the brain, rather than entering the C.N.S. by the normal sciatic nerve route. But when the vaccine was injected 48 hr. after the virus, although the effect upon susceptibility was considerably greater, the shift in distribution of symptoms was no longer evident; only one animal was found with cerebral symptoms, while nineteen showed paralysis. This apparently paradoxical finding is discussed below.

DISCUSSION

The course of a neurotropic virus infection may be modified by factors acting in at least two ways: first, by factors affecting the entry of virus into the C.N.S., and secondly, by factors affecting the course of virus proliferation within the C.N.S.

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In many cases, the route of entry of virus into the C.N.S. determines the subsequent distribution of symptoms, since the virus tends first to proliferate in and destroy those nerve cells nearest its point of entry. Evidence is presented above that the localization of paralysis in a limb inoculated with GDVII virus arises from this cause. Factors affecting the route of entry may therefore alter the distribution of symptoms. For example, the increase in the bulbar type of poliomyelitis in tonsillectomized children may be due to the surgical trauma making accessible to the virus an atypical portal of entry to the C.N.S. The experiments of Verlinde *et al.* (1955) on tonsillectomized monkeys indicate a direct neural migration of poliomyelitis virus from the damaged tonsillar region via the glossopharyngeal nerve to the medulla. Young mice show a greater tendency than adults to develop encephalitic symptoms when injected by a peripheral route with yellow fever virus (Theiler, 1930) or Western equine encephalomyelitis virus (Sabin & Olitsky, 1938); this may be associated with the high permeability of the 'blood-brain barrier' in young animals (Stern & Peyrot, 1927).

When the 'blood-brain barrier' is artificially lowered by intracerebral inoculation (Macklin & Macklin, 1920) or by other stimuli such as disturbances of osmotic pressure of the body fluids (Stern, Zeitlin & Gozman, 1928), one might expect both an increase in the relative incidence of encephalitic symptoms and an actual increase in susceptibility, when virus is introduced peripherally. Both effects were observed when intramuscular inoculations of GDVII virus were followed by intracerebral inoculations of vaccines and other substances, a result which further emphasizes the need to regard as relative such terms as 'neuronotropic' and 'blood-borne' applied to particular viruses. The facilitating effect of intracerebral or intrathecal inoculations upon infection with neurotropic viruses has been demonstrated by many previous workers (Flexner & Amoss, 1914, 1917; Nicolau & Galloway, 1928; Zwick, Seifried & Witte, 1929; Sawyer & Lloyd, 1931; Findlay & Elton, 1933; Webster & Clow, 1936; Lennette & Hudson, 1936; Burnet & Lush, 1938). King (1942) found that peripheral injection of osmotically active substances increased susceptibility to equine encephalomyelitis virus.

Disturbances of neurovascular relations could facilitate the entry of virus into parts of the nervous system other than the brain. It has been shown above that intramuscular injection of vaccines increases susceptibility to GDVII virus injected into the same muscle, and that intramuscular injection of vaccines or normal mouse brain suspension can influence the localization of paralysis in mice injected by other routes with GDVII or EMC virus. Bodian (1954) observed similar provoking and localizing effects of intramuscular inoculation in monkeys injected into the heart with poliomyelitis virus. He investigated the question, relevant also to our own results, of whether the intramuscular injections were acting locally to facilitate the entry of virus into damaged nerve fibres in the injected muscle, or were modifying, by some reflex action, the neurovascular relations of the corresponding anterior horn cells so as to allow direct entry into the spinal cord of virus circulating in the blood. Trueta & Hodes (1954) and Trueta (1955) present evidence that intramuscular inoculation of irritant substances can be followed by a profound localized alteration in the vascular state of the spinal cord, involving actual extra-

vasation of blood from the dural vessels and from vessels within the cord. They believe this to be the chief causative factor in induced localization of paralysis during poliomyelitis. Bodian also favours the direct vascular route to the spinal cord, on experimental grounds. On the other hand, the demonstration by Verlinde *et al.* (1955) that when diphtheria toxoid is injected into the muscle of one leg of monkeys infected orally with poliomyelitis virus, virus can be recovered from the sciatic nerve supplying the injected leg before it can be recovered from the C.N.S., is powerful evidence that in this case at least, virus was passing from the blood stream into damaged nerve fibres in the injected muscle, and then travelling to the spinal cord up the sciatic nerve.

If virus is already present in the nervous system, as in the case, for example, of intracerebral inoculation, the course of the disease can only be influenced by factors which modify the course of infection within the C.N.S. For those viruses, such as GDVII, whose entry into the C.N.S. appears always to be followed by overt signs of infection, such factors can affect incubation period or the distribution of symptoms, but not the incidence of infection. Thus intracerebral injection of vaccines into mice previously inoculated intracerebrally with GDVII virus was found to affect incubation period but not mortality; and Findlay & Howard (1950) claimed a similar effect of intravenous vaccine injections on incubation period, with mice inoculated intracerebrally with the TO strain of mouse encephalomyelitis.

Dean *et al.* (1951) demonstrated a marked localization of paralysis in a limb injected with vaccines when TO virus was injected into the brain. Here the vaccines could not have been influencing the portal of entry of virus into the nervous system, but must rather have modified the physiology of the corresponding anterior horn cells, causing a localized area of diminished resistance where virus could proliferate rapidly. That the physiology of anterior horn cells can be influenced by their peripheral connexions is suggested by the work of Howe & Bodian (1941), who showed that the susceptibility of anterior horn cells to experimental infection with poliomyelitis virus was lowered during the period of regeneration of the peripheral neurone. The localizing influence of exercise upon paralysis (Russell, 1947, 1949) might be due to a similar 'sensitizing' effect, since the work of Hyden (1943) indicates that muscular work alters the biochemical constitution of the anterior horn cells. Dean *et al.* (1951) found that the localizing effect of their vaccine injections was most marked when they were given anything up to a week earlier than the virus; so the negative results reported above on the effect of intramuscular vaccine injections in mice receiving GDVII virus into the brain may be due to the fact that the vaccine was injected later than the virus. Presumably the 'sensitizing' effect of peripheral trauma upon the anterior horn cells needs time to build up before the virus begins to proliferate in the C.N.S. The postulated 'sensitizing' effect affords an alternative explanation of the localization of paralysis in an injected limb when virus is injected by some other peripheral route. It also affords an explanation of the apparently paradoxical findings shown in Table 12. When vaccines are injected into the brain either immediately or 48 hr. after an intramuscular inoculation of GDVII virus, virus is enabled to pass directly from the blood into the brain, and an increase in susceptibility is observed. With the shorter

time interval, the predominating symptoms are, as expected, encephalitic in type; but with the longer time interval, virus entering the C.N.S. from the blood encounters anterior horn cells 'sensitized' by the intramuscular inoculation of 2 days previously, and localization of paralysis in the injected limb is observed.

SUMMARY

1. When GDVII virus was injected into the brains of mice, subsequent intracerebral injections of mixed pertussis and diphtheria prophylactic significantly shortened the incubation period. Subsequent intramuscular injections of mixed prophylactic had no influence on the course of the infection.

2. Intramuscular inoculation of either GDVII virus or EMC virus results in a marked localization of paralysis in the injected limb. With GDVII virus, the localization probably arises from the use of the sciatic nerve route for entry into the C.N.S., and is unaffected by injection of substances into the opposite limb. With EMC virus, localization can be modified by injections into the opposite limb.

3. When GDVII or EMC virus was injected by a peripheral route other than intramuscular, intramuscular injections of vaccines or normal mouse brain suspension induced localization of paralysis in the injected limb.

4. Intramuscular inoculation of vaccines increased mortality in mice injected into the same limb muscle with GDVII virus. Within the limits tested the later the vaccines were injected relative to the virus, the greater was the effect.

5. When the vaccines used were more than a few months old, this facilitating effect was replaced by a protective effect. Mice injected intramuscularly with aged vaccines were significantly more resistant than the controls to GDVII virus injected into the same muscle.

6. Intracerebral inoculation of vaccines, and to a lesser extent other substances, increased mortality in mice which had received intramuscular inoculations of GDVII virus, by allowing virus to pass directly from the blood into the brain. This resulted in a predominance of cerebral symptoms when the vaccines were injected immediately after the virus, but not when the interval was lengthened to 48 hr.

7. The mode of action of modifying factors in neurotropic virus infections is discussed in the light of these results.

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