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The virulence of strains of poliovirus for cynomolgus monkeys after subcutaneous injection

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INTRODUCTION

In comparing the neurovirulence for monkeys of attenuated strains of poliovirus, the intrathalamic and intraspinal routes of injection have been widely used (Sabin, 1959), and recently the intramuscular route has also been shown to be of value (Kirschstein, Borman, Baron, Friedman, Murray & Hottle, 1960). In this laboratory the subcutaneous route has been found to be a satisfactory method of infecting monkeys with the attenuated Brunenders strain of Type 1 poliovirus (Coid, Tobin & Beswick, 1960), and it was considered that this route might also be used in comparing the virulence of different strains. Experiments were therefore made, in which strains of all three types were tested by the subcutaneous injection of monkeys. The experiments included an examination of the production of (*a*) paralysis, (*b*) lesions of the central nervous system, (*c*) viraemia and (*d*) specific antibody. In addition, for the Type 1 strains, a comparison was made between the subcutaneous and intramuscular routes of injection.

MATERIALS AND METHODS

Virus strains

The strains of virus used were the Sabin attenuated Types 1, 2 and 3, Cox attenuated Types 1, 2 and 3, the Brunenders Type 1 partially attenuated strain, MEF-1 (Type 2) and Saukett (Type 3).

(*a*) *Sabin Types 1, 2 and 3.* These were subcultures from batches of oral vaccine prepared by Dr A. B. Sabin, and were received from Dr P. Cooper of the Medical Research Council Laboratories, Carshalton. Three passages at 33° C on monolayer monkey kidney tissue cultures were made—the first in the presence of 5 % pooled monkey serum and the other two in 199 medium alone.

(*b*) *Cox Types 1, 2 and 3.* The virus suspensions, received directly from Dr V. Cabasso of the Lederle Laboratories, New York, were used in the experiments without further subculturing.

(*c*) *Brunenders (Type 1), MEF-1 (Type 2) and Saukett (Type 3).* Each of these three strains were received originally from Dr W. Wood of the Glaxo Laboratories Ltd., and had been passaged six times on monkey kidney cell cultures at 37° C.

Injection of virus

Cynomolgus monkeys (*Macaca irus philippinensis*), weighing 1·4–2·4 kg. were used throughout. The subcutaneous injections were given about 2 cm. from the lateral aspect of either nipple. In the experiments in which virus was given intramuscularly, injections were made into the right or left deltoid muscle. The dose of virus injected is shown in Table 1, and was determined by titration on monkey kidney cell cultures, using ten tubes for each 10-fold dilution of virus. The volume of inoculum was 1 ml. in the experiments with the Type 1 strains, and 2 ml. in those with Types 2 and 3.

Isolation of virus from blood serum

Blood samples were taken from the femoral vein at various intervals during the experiments, or from the heart at the time of death. The serum samples were examined for virus on monkey kidney cell cultures by the methods previously described (Coid *et al.* 1960), except that in the present experiments only 0·5 ml. of each sample was tested.

Antibody titrations

Serum samples were titrated for poliomyelitis neutralizing antibodies by the galactose colour test (Mussett, Dimic, Ege & Tobin, 1961) and, in the first intramuscular experiment, on monolayer monkey kidney cell cultures (Biological Standards Control Laboratory, 1957).

Histological examination

At the end of the observation period, which varied from 23 to 26 days, the animals were anaesthetized deeply with pentobarbitone sodium, perfused with formol saline and the brains and spinal cords removed for histological examination (Biological Standards Control Laboratory, 1957). The following levels were examined in each monkey:

Lumbar enlargement (L4-S1)	10 blocks
Thoracic cord (T2-L3)	3 blocks (2nd intramuscular experiments); 15 blocks (All subcutaneous experiments)
Cervical enlargement (C5-T1)	10 blocks
(C1)	1 block
Medulla	3 blocks (The highest level including roof nuclei and dentate nuclei)
Midbrain (including red nuclei and IIIrd nerve nuclei)	1 block
Thalamus and basal ganglia (right and left)	2 blocks
Sensory and motor cortex (right and left)	2 blocks

The tissues were embedded in paraffin, cut at 15μ, and stained by Einarson's gallocyanin technique (Beswick, 1958).

The grading of severity of the lesions in individual animals is based on a scoring system similar to that used by Melnick & Brennan (1959). As with their method, individual hemisections of the cord are graded from 0 to 4. Grade 0 indicates no specific lesion seen. Grade 1 corresponds to Grades 1 and 2 of Melnick & Brennan and represents the presence of one or two glial foci with or without some mild perivascular cuffing of nearby vessels. Grade 2 represents more extensive inflammatory infiltration, but without obvious loss of neurones. Grade 3 represents obvious, partial destruction of neurones regardless of the intensity of the cellular infiltration and Grade 4 complete, or almost complete, loss of anterior horn cells. If ten levels are examined from one enlargement, the maximum score is 80. Scores 1–15 correspond to the category 'mild' in Table 3, scores of 16–40 to the category 'moderate' and scores of 41–80 to the category 'severe'. The grading of the intramuscularly inoculated animals is derived from the scores of the cervical enlargements; that of the subcutaneously inoculated monkeys from the means of the scores of lumbar and cervical enlargements.

RESULTS

The incidence of viraemia, of paralysis and of histologically evident lesions in the central nervous system are recorded in Table 1 and the antibody levels in Table 2. The histological findings are given in more detail in Table 3.

Viraemia

Type 1 strains

It is evident that virus was isolated less frequently throughout the experimental period from monkeys injected, either subcutaneously or intramuscularly, with the Sabin strain than from those in corresponding experiments injected with the Cox or Brunenders strains. Viraemia was not observed later than three days after injection in any monkey given the Sabin strain by either route; only those injected with the Cox strain showed evidence of viraemia at 10 days. Those showing viraemia 6–8 days after injection also showed it at 3 days, except in one instance where virus was isolated for the first time 8 days after the animal had received the Cox strain subcutaneously.

Type 2 strains

It is clear that virus was isolated from a greater number of animals which received the MEF-1 strain than from those injected with either of the other two strains. Those monkeys showing viraemia at 6–8 days also showed it at 3 days.

Type 3 strains

Virus was not demonstrated in the sera of any animals injected with the Cox strain. At 3 days after injection, only one monkey of those injected with the Sabin strain and two of those given the Saukett strain had viraemia.

Table 1. *The virulence of poliovirus strains for cynomolgus monkeys*

Virus Type	Strain	Route of injection	Dose of virus TCID 50 (Log_{10})	No. of monkeys injected	No. of monkeys with viraemia (days after injection)			No. of monkeys with paralysis	No. of monkeys with C.N.S. lesions
					3	6–8	10		
1	Cox	Subcutaneous	7·2	10	3	2	1	0	2
		Intramuscular (1)	7·2	10	6	2	0	0	6
		Intramuscular (2)	7·2	10	7	3	3	0	4
	Sabin	Subcutaneous	7·3	10	1	0	0	0	0
		Intramuscular (1)	7·3	10	1	0	0	0	10
		Intramuscular (2)	7·3	10	2	0	0	0	3
	Brunenders	Subcutaneous	7·0	10	8	0	0	0	1
		Intramuscular	7·0	10*	8	2	0	0	3
2	Cox	Subcutaneous	7·1	9	1	0	0	2	5
	Sabin	Subcutaneous	6·9	10	1	1	0	0	0
	MEF-1	Subcutaneous	6·7	9	6	2	0	3	3
3	Cox	Subcutaneous	6·8	10	0	0	0	0	0
	Sabin	Subcutaneous	7·5	10	1	0	0	0	0
	Saukett	Subcutaneous	7·0	10	2	0	0	0	0

* One monkey died 5 days after injection with purulent meningitis.

Histological findings

One animal inoculated with the Brunenders strain showed apparent paralysis before it died on the fifth day after inoculation; examination revealed severe purulent meningitis, but no evidence of poliomyelitis. Two animals, one inoculated with Cox Type 2 strain and one with MEF-1, failed to recover from the anaesthetic and were not examined histologically.

Table 2. *The geometric mean neutralizing antibody titres of sera from cynomolgus monkeys injected with different strains of poliovirus*

Virus Type	Strain	Route of injection	Titre (days after injection) 3	6–8	10	18	23–26
1	Cox	Subcutaneous	0	3	3	60	239
		Intramuscular (1)	0	2	4	—	—
		Intramuscular (2)	0	3	6	34	81
	Sabin	Subcutaneous	0	9	43	465	676
		Intramuscular (1)	0	4	30	—	—
		Intramuscular (2)	0	11	69	194	161
	Brunenders	Subcutaneous	0	7	39	955	1351
		Intramuscular	0	7	35	146	256
2	Cox	Subcutaneous	0	10	23	91	63
	Sabin	Subcutaneous	0	4	8	95	128
	MEF-1	Subcutaneous	0	14	37	645	1100
3	Cox	Subcutaneous	2	18	30	97	37
	Sabin	Subcutaneous	2	6	23	24	49
	Saukett	Subcutaneous	1	2	15	34	97

The dose of virus given to each group of monkeys is shown in Table 1.

Of twenty-six animals having poliomyelitis lesions after intramuscular inoculation (deltoid muscle) all had involvement of the cervical cord. However, the proportions showing involvement of the lumbar cord as well differed with the three strains: Cox Type 1, 7/10, 70%; Sabin Type 1, 6/13, 50%; Brunenders Type 1, 3/3, 100%.

The occurrence of poliomyelitis lesions in all ten animals inoculated with Sabin's Type 1 virus in the first intramuscular experiment and in only three of the ten in the second experiment gives some indication of the variation which is likely to be observed between different tests, using this route.

Lesions graded as severe were only observed in monkeys inoculated with Cox Type 2 and MEF-1. Sabin's Type 1 strain produced only mild lesions after intramuscular inoculation and none of his strains produced any lesions after subcutaneous inoculation.

In only one of the histologically positive animals was there any doubt about the specificity of the lesions.

Table 3. *Summary of the histological findings in cynomolgus monkeys injected with different strains of poliovirus*

Virus Type	Strain	Route of injection	No. of monkeys injected	Histological findings: No. of monkeys showing polio lesions in: Lumbar enlargement	Thoracic cord	Cervical enlargement	Brain	Total no. of monkeys with lesions	Intensity of lesions: Mild	Moderate	Severe
	Cox	Subcutaneous	10	2	2	2	2	2	1	1	0
		Intramuscular (1)	10	3	—	6	4	6	2	4	0
		Intramuscular (2)	10	4	3	4	4	4	0	4	0
	Sabin	Subcutaneous	10	0	0	0	0	0	—	—	—
		Intramuscular (1)	10	5	—	10	4	10	10	0	0
		Intramuscular (2)	10	1	0	3	1	3	3	0	0
	Brunenders	Subcutaneous	10	1	1	1	1	1	0	1	0
		Intramuscular	10	3	2	3	3	3	1	2	0
2	Cox	Subcutaneous	9	5	5	5	5	5	1	1	3
	Sabin	Subcutaneous	10	0	0	0	0	0	—	—	—
	MEF-1	Subcutaneous	9	3	3	3	3	3	0	2	1
3	Cox	Subcutaneous	10	0	0	0	0	0	—	—	—
	Sabin	Subcutaneous	10	0	0	0	0	0	—	—	—
	Saukett	Subcutaneous	10	0	0	0	0	0	—	—	—

Paralysis

Paralysis occurred only in the groups of monkeys injected with the Cox Type 2 and MEF-1 strains. Of the two monkeys which became paralysed with the Cox strain, one had complete paralysis in both legs and the other partial paralysis in one. A third monkey died suddenly overnight and was not observed to have paralysis, but histological examination showed extensive and severe lesions of poliomyelitis.

Two of the three monkeys which were paralysed with the MEF-1 strain had partial paralysis in one leg and the third was partially paralysed in both.

Antibody response

Type 1 strain

The least rapid rise in antibody was observed in monkeys injected with the Cox strain. It can be seen that the responses of the groups injected with the Sabin and Brunenders strain were similar and both showed a markedly higher titre 10 days after injection than those which were given the Cox strain.

Type 2 strain

The least rapid antibody response during the first ten days was observed in monkeys injected with the Sabin strain, but the mean level of antibody produced by this strain from the 18th day was similar to that produced by the Cox strain. The antibody level following injection of the MEF-1 strain was much higher at 18 and 23 days than that produced by either of the other two strains.

Type 3 strains

There was little difference between the antibody responses to the three strains, except at 7 days, when the titre of the group injected with the Cox strain was slightly higher than that of the other two.

DISCUSSION

It has been shown with three Type 1 strains of attenuated poliovirus that the proportion of monkeys with lesions of the central nervous system was greater following intramuscular injection than that after injection by the subcutaneous route. The number of animals in each group is too small for differences in the incidence of poliomyelitis lesions produced by the three Type 1 strains injected intramuscularly to be significant. However, the fact that the highest incidence occurred with Sabin Type 1 (65%) and the lowest with Brunenders (30%) is surprising. It is generally accepted that the neurons of the lumbar cord are more susceptible to poliomyelitis than are those of the cervical cord. Nevertheless, all the animals with lesions after intramuscular inoculation showed lesions in the cervical cord, but only sixteen of twenty-six animals also showed lesions in the lumbar cord. This observation lends support to the contention of Kirschstein *et al.* (1960) that virus spreads from the site of an intramuscular injection to the spinal cord along the regional nerves rather than by way of the blood-stream.

After subcutaneous inoculation all animals with poliomyelitis lesions, whatever the virus injected, showed involvement of both spinal cord enlargements and of the thoracic cord. Comparison of the pattern of viraemia showed that the Sabin strain of Type 1 differed from the other two in that it produced viraemia in fewer animals 3 days after injection, and beyond this time virus was not isolated from the sera of any monkeys injected with this strain. On examination of the antibody titres, it was apparent that the Cox strain of Type 1 differed from the Sabin and Brunenders strains, both of which produced a higher titre at corresponding intervals after infection.

The Sabin strain of Type 2 virus was the only one of the three of this type which did not cause central nervous system lesions and paralysis in a proportion of the monkeys injected, and during the first 10 days produced a slower antibody response. The Cox Type 2 and MEF-1 strains could not be differentiated on the basis of C.N.S. lesions and paralysis, but the MEF-1 strain caused viraemia in a higher proportion of animals, and at 18 and 25 days the antibody titre was considerably higher than that induced by either the Cox or Sabin strains.

There was little obvious difference between the three strains of Type 3, except that at seven days after injection the Cox strain produced a slightly higher antibody titre.

From these observations it may be concluded that the subcutaneous route of injection offers a promising method for comparing and characterizing strains of poliovirus.

SUMMARY

The virulence of strains of the three types of poliovirus have been examined by the subcutaneous injection of monkeys. The strains used were the Sabin attenuated Types 1, 2 and 3, Cox attenuated Types 1, 2 and 3, the Brunenders Type 1 partially attenuated strain, MEF-1, Type 2 and Saukett, Type 3.

The results obtained indicated that the subcutaneous route of injection offers a promising method for comparing and characterizing strains of poliovirus.

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