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PRESENT POSITION OF IMMUNIZATION AGAINST POLIOMYELITIS WITH LIVE VIRUS VACCINES*

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Immunization against poliomyelitis with orally administered live attenuated poliovirus vaccine presents special problems not previously encountered with other live virus vaccines employed in human beings, and it is entirely proper to approach it in the most cautious and critical manner. The recent communications (Dick and Dane, 1958; Dane *et al.*, 1958; Clarke *et al.*, 1958) and editorial comments (leading article, 1958) in the *British Medical Journal* did not take into account all the available published and unpublished data that were available to the writers, and, in my opinion, did not give a complete picture of the present status of our knowledge on this subject.

Accordingly I am prompted to bring up to date the information available to me on four topics that came under special consideration in the recent discussions: (1) neurotropism of viruses excreted by the vaccinated individuals and after further multiplication in contacts; (2) persistence of antibody and its relation to resistance of the intestinal tract to reinfection; (3) effect of multiple doses of Salk vaccine and of high levels of neutralizing antibody produced by killed virus vaccine on multiplication of poliovirus in the pharynx and intestines of children; and (4) the present status of field trials with the large lots of the three types of vaccine that I prepared two years ago.

Problems in Estimation of Neurotropism of Polioviruses

In working out the quantitative aspects of neurotropism of various polioviruses for monkeys, and its relationship to the neurotropism of the same viruses for chimpanzees and, by inference based on epidemiological data, also for man, I found that the thalamic neurones of the monkey are more resistant than those in the lumbar enlargement of the spinal cord, and that the lumbar neurones of chimpanzees are much more resistant than those of monkeys (Sabin, 1956a, 1957a). Since the neurotropism of a poliovirus particle is in effect a measure of its capacity to multiply sufficiently in a given neurone to be able to spread to and destroy the large number of other neurones required to produce clinical manifestations, it is important not only to differentiate between the effect of 100 and 100,000 or 100,000,000 infective virus particles, but also to take into account the precise placement of the inoculum. During

the past six years I have used certain techniques of intracerebral and intraspinal inoculation on thousands of monkeys which yielded sufficiently reproducible results to permit a fair quantitative estimate of neurotropism and gradation of strains (Sabin, 1956a, 1957b). Although I could have imagined, *a priori*, that certain variations in technique might yield different results, I did not realize until recently how important those differences could be in the estimation of both "cerebral" and "spinal" neurotropism.

"Cerebral" Neurotropism

In measuring "cerebral" neurotropism, one attempts to measure the capacity of poliovirus particles to multiply sufficiently in the neurones of the thalamus and adjacent susceptible regions to permit further progression along neural pathways to the motor neurones of the spinal cord, whose destruction in sufficient numbers to produce clinical paralysis serves as the final index of activity. If, however, the inoculum itself causes a sufficiently extensive traumatic lesion to lay open the tracts leading directly to the spinal cord, enough virus can invade the spinal neurones without the necessary preliminary multiplication in the susceptible cerebral neurones. Confirmation of such an event has been obtained by showing that the virus recovered from the spinal cord of such monkeys has no greater intracerebral activity than that used for the original inoculation (Sabin, 1955a).

Before tissue cultures became available, the technique of intracerebral inoculation that was most suitable for detecting minimal amounts of poliovirus involved the injection of 0.5-ml. quantities into each thalamic region (Bodian *et al.*, 1950). This technique produces extensive local damage and is often associated with varying clinical manifestations resulting from initial damage and subsequent inflammatory reaction in the tracts and adjacent regions; it is also most likely to permit mechanical invasion of the spinal neurones, and is therefore not suitable for an estimate of "cerebral" neurotropism. This is the technique that was used by Clarke *et al.* (1958) in their tests on excreted virus, and by others. So long as the virus used has a very limited "spinal" neurotropism, this procedure will not give rise to spinal paralytic manifestations, although the interpretation of a paresis based on cerebral trauma and subsequent inflammation may be confused by the presence of limited focal, specific poliomyelitic lesions in

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the spinal cord, which by themselves are insufficient to give rise to spinal paralytic manifestations. However, when a virus of greater "spinal" neurotropism is being tested this procedure is most likely to give rise to misleading results.

In my own work with viruses of low "spinal" activity, I have usually inoculated 1 ml. into the right thalamic region, which is less traumatic than 0.5 ml. given into each side, but still is not infrequently associated with traumatic hemiparesis, or hemiplegia, or a lower facial paralysis of the cerebral type. For all the cerebral tests on excreted virus that I reported previously, I used only 0.5 ml. on one side. Since it is possible to inoculate 10 million or more tissue culture infective doses in a volume of 0.2 ml. some have suggested that a still smaller inoculum might be preferable. The danger in decreasing the volume too much is that one might more readily miss the thalamic region. Although the practice of inserting a 2.5-cm. 24-gauge needle about 6 mm. from the midline along the coronal suture usually places the inoculum in the right place, this is not an invariable guide. It is most important to pull back on the piston of the syringe to make sure that ventricular fluid is not obtained prior to inoculation and that the inoculum cannot be pulled back after inoculation; if the latter occurs it is important to change the position of the needle and reinoculate until it is evident that the fluid has diffused in the brain tissue and cannot be recovered.

Another worth-while precaution for avoiding unnecessary trauma is to inject slowly and not create a jet stream by forceful ejection of the fluid. It should also be stressed that an erroneous impression of lack of "cerebral" neurotropism might be obtained if the needle is inserted too far from the midline, and the inoculum is not placed in the vicinity of an area containing neurones that are susceptible to polioviruses.

In testing for cerebral neurotropism it is also important to remember that in mixed populations of virus particles the undiluted culture fluid may yield negative results, while paralysis may show up in the animals inoculated with diluted material (Sabin *et al.*, 1954; Sabin, 1955a). In 1953 I demonstrated such interference experimentally by inoculating a mixture of 100,000 tissue culture infective doses of an attenuated type 1 virus with 10 intracerebrally paralytic doses of the virulent Mahoney virus. Only 4 of the 10 monkeys inoculated with the mixture developed paralysis, while all 5 receiving the small dose of Mahoney virus succumbed and all 9 receiving only the attenuated virus remained well.

In interpreting the results of an intracerebral inoculation, particularly when only a small proportion of the inoculated monkeys exhibit spinal paralysis, with quantitatively compatible specific lesions, one must also keep in mind not only that the monkey nervous system acts as a selective medium for the small proportion of more neurotropic virus particles that may be present in a large population, but also that more neurotropic mutants, sometimes strict neurotropic mutants, not originally present in the inoculated material, may arise during the course of viral multiplication in the brain (Sabin, 1955a). I have observed this repeatedly and reported it on several occasions, particularly with viruses which may still multiply to a certain extent in the cerebral neurones.

Tests in tissue culture reveal the presence of strict neurotropic mutants, either by the failure of isolating

cytopathogenic virus from the spinal cord, or by the zone phenomenon in which a 10% extract of the spinal cord produces no cytopathogenic effect or a very delayed one, while higher dilutions produce a rapid effect. Abortive infections have also been encountered in which the spinal cord has no demonstrable virus by passage either in tissue culture or in intracerebrally or intraspinaly inoculated monkeys. It is noteworthy that I have not observed any of these phenomena, pointing to mutation in the nervous system of the monkey, in large numbers of monkeys that were inoculated intracerebrally with the strains of limited spinal activity that I finally selected for inclusion in the vaccine to be used for more extensive trials in man (Sabin, 1957b).

"Spinal" Neurotropism

The measure of "spinal" neurotropism is also not a measure of an "all or none" property, but of the varying capacity of poliovirus particles to multiply sufficiently in the motor neurones at the site of inoculation in the spinal cord to permit spread to the large enough number of contiguous neurones required for the production of various degrees of paralysis. It is well known that a muscle, indeed an individual muscle fibre, receives its motor innervation from neurones at multiple levels in the spinal cord, and that rather extensive lesions can be produced mechanically or specifically by virus action, without noticeably affecting the capacity of a muscle to function, if these lesions are not widely distributed over many levels. Thus, the requirements of this method are, on the one hand, to place the inoculum close enough to the motor cells to be infected, and, on the other hand, to avoid the traumatic destruction, by the initial disintegration of tissue and subsequent inflammatory response, of so many neurones that the further specific viral attack on a relatively small number of additional neurones will be sufficient to produce clinical manifestations varying from weakness to complete paralysis. The problem here is quite different from that involved in searching for an isolated particle of virulent virus in the safety testing of a killed-virus vaccine, where volumes as large as 0.5 ml. are purposely inoculated in a manner to produce traumatic paralysis in most of the monkeys, because the effect is then usually evident by the subsequent extensive spread of the paralysis.

The method that I have used in the past six years has permitted the demonstration of different grades of "spinal" neurotropism in strains of poliovirus that were completely or almost completely devoid of "cerebral" neurotropism for the monkey. It was the basis of all the work over a period of several years by which many strains were eliminated and only a few retained as candidates for a vaccine, ultimately designed to be tested on a large scale (Sabin, 1956a, 1957a, 1957b). Viruses which in small doses regularly produced paralysis after spinal inoculation by the method that I used obviously were capable of greater spread to other neurones than those that would do so only after inoculation of a 1,000 to 10,000 times larger dose. The viruses that I finally selected had such limited spinal activity, by this method of inoculation, that even amounts in the range of 1 million or 10 million tissue culture infective doses failed in most monkeys to produce any paralysis and in occasional ones even failed to produce specific lesions. However, the majority of clinically well monkeys showed limited focal specific lesions, indicating that the viruses multiplied to a limited extent, but in an

abortive fashion which prevented involvement of the large numbers of neurones required for the production of clinical manifestations. In chimpanzees these same viruses in about 10 million tissue culture infective doses produced neither paralysis nor lesions after inoculation into the grey matter of the lumbar cord.

During recent months it became apparent to me, however, that "die Methode ist alles," and I therefore find it necessary to describe in some detail the method that I used and to indicate how certain variations can, on the one hand, give rise to an erroneous impression of low or absent "spinal" neurotropism, and, on the other, to an impression of much greater neurotropism. By dissection and by inoculation of dyes, I decided that in monkeys the lumbar enlargement can be located by counting the four spaces between the spinous processes that lie above the level of the iliac crests (see Fig. 1). This fourth space corresponds also to the first space below the level of the last rib, and this may be used in checking the exact position. In training people I find that they most frequently mistake the third space for the

fourth, which, as I shall show later, turns out to be crucial. The lumbar cord is so small and the interval between the spaces relatively so large, that a mistake in one space too low places the inoculum in the region of the lumbosacral conus, where it may readily diffuse into the roots rather than into the grey matter.

The monkey is tied on a board, face

FIG. 1.—Diagram for location of lumbar enlargement of spinal cord of monkey where virus is inoculated for evaluation of "spinal neurotropism."

down, and a stiff cardboard roll (about 7.5 cm. in diameter) is placed under the abdomen to arch the back. I selected a 20-gauge needle (1.5 cm. long, short bevel) for inoculation simply because it was firm enough to penetrate the skin without bending on bony obstructions, but this also turns out to be important. The needle, attached to a tuberculin syringe, is passed through the fourth space, slightly to one side of the midline, until it hits the bone on the ventral aspect of the spinal canal, making certain that a contraction is observed in at least one group of muscles of one leg; if such a contraction or kick is not observed the needle is withdrawn and the procedure is repeated until a definite kick is observed. The needle is then withdrawn 1 to 2 mm. (depending on the size of the monkey) and 0.1 ml. is inoculated very slowly, without changing the position of the needle or attempting to produce additional muscle contractions. Great care is taken to avoid forcible injection which might give rise to a jet stream and greater disintegration of tissue. Even 0.1 ml. represents a large proportion of the total volume of the grey matter in the lumbar cord, and some of it must of necessity leak out around the cord or too much of the grey matter would be destroyed.

Figs. 2a and 3a show the type of lesions usually produced by the inoculum as seen 21 days after inoculation in monkeys, which at no time exhibited any signs of muscle weakness. The lumbar cord is cut at approximately 1-mm. intervals (one side being marked

FIG. 2.—Lumbar cord of monkey inoculated intraspinally with 10^6 TCD₅₀ of type 1, L Sc, 2 ab virus. (a) Arrows show inoculation scar leading into anterior horn, which shows no poliomyelitis lesions; (b) another level of lumbar cord with arrow pointing to residue of inoculum—note complete absence of inflammatory response or poliomyelitic lesions.

FIG. 3.—Lumbar cord of monkey inoculated intraspinally with 10^6 TCD₅₀ of type 1, L Sc, 2 ab virus. (a) Arrow on right points to disintegration of tissue in posterior and anterior horns produced by inoculum, and arrow on left points to focal infiltration—note predominantly normal roots; (b) adjacent level of lumbar cord of same monkey—note complete absence of lesions.

with indian ink) and approximately 20 levels are examined histologically in each monkey. It is noteworthy—and this needs to be stressed—that by the method described above the inoculum can usually be located in only 1 of these 20 levels; when traumatic paralysis is apparent within 24 hours after inoculation, the inoculum can be located at several levels. Occasionally the inoculum cannot be located, but a root lesion indicative of destruction of neurones somewhere can be found. Occasionally also the inoculum fails to reach the grey matter, and the needle track can be seen in the lateral white matter (Fig. 4); in such cases the absence of clinical manifestations or specific lesions is of no significance in the final evaluation of the test.

In Fig. 2a the damage from the inoculum is minimal, but the needle track can be seen passing through the grey matter of the posterior horn up to the anterior horn. This particular monkey was inoculated with approximately 10^5 TCD₅₀ (50% tissue culture infective doses) of the type 1 vaccine (L Sc, 2 ab strain), and

FIG. 4.—Lumbar cord of monkey inoculated intraspinally with 10^7 TCD₅₀ of type 1 "Chat" virus—arrow points to inoculation damage in lateral white matter, and note complete absence of lesions in grey matter. This was the only one of 10 monkeys, inoculated with the same dose, that failed to develop paralysis. Since monkeys that received only 10^8 TCD₅₀ of the same virus into the grey matter all developed paralysis, this illustrates the innocuity of even large doses of intracerebrally avirulent virus except when it is placed in direct contact with susceptible neurones.

no specific lesions are discernible even in the anterior horn cells adjacent to the inoculum, and all the other levels of the lumbar cord from the same monkey were as devoid of specific lesions as that shown in Fig. 2b. In Fig. 3a the inoculation damage involves disintegration

FIG. 5.—High power of infiltrated zone in right anterior horn of Fig. 3a—note intact neurones.

of the grey matter of most of the posterior and anterior horns at this level with slight focal infiltrative lesions on the opposite side. Actually in the midst of the inflammatory reaction in the destroyed anterior horn one can find many shrunken but architecturally intact neurones (see Fig. 5). Despite this evidence of the perfect placement of the inoculum, which in this instance contained about 10^6 TCD₅₀ of the same type 1 vaccine, all the other levels of the cord from the same monkey were as devoid of lesions as that shown in Fig. 3b. As previously pointed out, in most instances one can find evidence of either contiguous or scattered focal specific lesions elsewhere in the lumbar cord, and only exceptionally slight focal lesions in the cervical cord, of the monkeys that remain well after spinal inoculation of the three selected vaccine strains.

It is obvious that there is a certain amount of variation in the response of individual monkeys which is not dependent on the accuracy of the placement of the inoculum. Within the limits of these slight variations, however, this method has yielded reproducible

TABLE I.—Effectiveness and Reproducibility of Described Procedure for Comparing Spinal Neurotropism of Attenuated Polioviruses in Monkeys

Type 1 Strains							Type 3 Strains			
TCD ₅₀ Inoculated Log 10	Brunhilde- Enders- Chimpanzee	“ Chat ” Koprowski (Large Lot)	L Sc, 2 ab (Large Lot)				TCD ₅₀ Inoculated Log 10	Leon, 12 a ₁ b (Large Lot)		“ Fox ” Koprowski (Large Lot)
			Test 1	Test 2 (Rhesus)	Test 3			Test 1	Test 2 (Rhesus)	
					5-8 lb. Monkeys	2-5-4 lb. Monkeys				
7.0-7.2	Not tested	9/10	12/10	2sl+22/10	2+3sl/10		6.9	1+12/10	1*,10	
6.0-6.2		3/4	5/5	12/5	3/5	1sl/5	5.9-6.1	0/5		9/10
5.0-5.2		2/3	5/5	12/5	0/5	0/5	4.9-5.1	0/5		4/5
4.0-4.2		4/4	3/5				3.9-4.1			5/5
3.0-3.2		3/4	4/5				2.9-3.1			3/5
2.0-2.2		2/4	Not tested							

Note.—All tests in cynomolgus monkeys of about 5 to 6 lb. (2.2 to 2.7 kg.) except when otherwise indicated. Numerator=number of monkeys showing paralysis of varying extent with onset two days or later after inoculation confirmed by histological evidence of specific poliomyelitic, neuronal lesions of sufficient extent to account for clinical manifestations. Denominator=Number of monkeys inoculated and surviving at least 14 days. Sl=Only limited clinical involvement giving rise to localized weakness or transitory partial paralysis of one leg. ?=Involvement only of toes or localized subsequent extension of traumatic partial paralysis first seen within 24 hours after inoculation, associated with only limited, focal poliomyelitic lesions of insufficient extent to account for clinical manifestations.

* This monkey first exhibited weakness of one foot 17 days after inoculation and progressed to partial paralysis of the leg the next day and no further by the 20th day, when it was sacrificed for study; despite the presence of specific poliomyelitic lesions no virus could be recovered in tissue cultures of various dilutions of an extract of the spinal cord.

Note on Histological Check.—In the few monkeys that failed to show paralysis after inoculation with the "Brunhilde-Enders-Chimpanzee" (excepting one monkey in the $10^{8.2}$ group), "Chat," and "Fox" Koprowski strains, the inoculum was either found in the lateral white matter (Fig. 4) or could not be located in the sections. In the monkeys that failed to show paralysis after inoculation with the "L Sc, 2 ab" and "Leon, 12 a₁b" strains the inoculum, with only few exceptions, was located in the grey matter as shown in Figs. 2 and 3.

results with the same or similar material, in both cynomolgus and rhesus monkeys, as indicated in my previous publications (Sabin, 1956a, 1957a, 1957b) and in Table I. It is obvious from the results obtained with more neurotropic strains such as the Brunhilde-Enders-Chimpanzee (the so-called Brunenders strain used in the British killed-virus vaccine), the "Chat," the "Fox" type 3, and others (Sabin, 1955a), that inocula in the range of 10^2 and 10^3 TCD₅₀ can produce paralysis despite the fact that the entire inoculum does not remain at the inoculated site by my procedure of spinal injection. Thus a strain that produces less paralysis at 10^7 TCD₅₀ than another at 10^3 TCD₅₀ can be regarded as being 10,000 times more attenuated.

I have been led to investigate the effect of certain deviations from this procedure because of differences in results obtained in some studies during the past year. I have previously reported that cultures of virus excreted in the stools of adults and children who were fed the three selected vaccine strains usually showed a definite increase in "spinal" neurotropism (Sabin, 1957b). Verlinde *et al.* (1958), using aliquots of the same large lots of vaccine, found practically no "spinal" neurotropism in tests on cultures of a large number of early and late stools obtained from children who were fed the type 1 and type 2 vaccines. During the past year I again confirmed my original observations in tests on another group of 10 children who received the same type 1 vaccine, and it appeared highly improbable that chance alone was responsible for the difference in our results.

Professor Verlinde informed me that he used a procedure essentially similar to mine except that he put his needle in slowly until he saw cerebrospinal fluid well up, at which time he pushed it in deeper and attached the syringe for inoculation of the virus. In tests on a number of monkeys I found that I could not obtain cerebrospinal fluid when I put the needle in the fourth space above the level of the iliac crests, and could first obtain it only in the third space, which is well below the lumbar enlargement of the spinal cord. I then selected a stool culture of type 1 excreted virus which produced non-progressive paralysis in a dose of $10^{5.4}$ TCD₅₀, and also partly in a dose of $10^{4.4}$ TCD₅₀, and inoculated 12 monkeys intraspinally with the $10^{5.4}$ TCD₅₀ in different ways, although in each instance the needle was inserted in a manner to produce a distinct muscular contraction in one limb.

None of the 12 monkeys exhibited any evidence of traumatic paralysis within 24 hours after inoculation. The four monkeys which received this inoculum in the third space, withdrawing the needle somewhat after it hit the ventral body surface of the canal, all remained well; the four monkeys which were inoculated in the fourth space rather close to the ventral bony surface (putting the inoculum almost entirely in the anterior horn) all developed moderate paralysis, three of them only in one leg; of the four monkeys that were also inoculated in the fourth space but with the needle withdrawn about 2 mm. from the ventral bony surface only two exhibited a slight partial, non-progressive paralysis. It seems highly probable, therefore, that the different results obtained by Verlinde *et al.* (1958) may be explained on the basis of placing the inoculum too low in the spinal cord. That Verlinde's method of spinal inoculation can yield positive results with virus of somewhat higher neurotropism is evident from his data on some of the type 3 excreted virus.

A similar explanation probably obtains for the difference in the results that I got in the tests on the large lots of Koprowski's type I (Chat) and type 3 (Fox) vaccine that were used in the Belgian Congo trials (Courtois *et al.*, 1958). In the tests carried out by Koprowski's associates these viruses produced paralysis in only an occasional monkey inoculated intraspinally with the largest doses, while in the tests that I carried out by my standard method the results shown in Table I were obtained. Only 4 of the 55 monkeys used in my tests on the Koprowski strains had some limited traumatic paralysis within 24 hours after inoculation; with few exceptions, the paralysis that developed subsequently was extensive and often involved both lower extremities. Sufficiently extensive specific poliomyelitic lesions to account for these clinical manifestations were found in the lumbar cord, and to a less extent usually also in the cervical cord and medulla. It is obvious from this study that comparisons of neurotropic activity of different strains have significance only with reference to the methods used in the tests.

Dr. J. L. Melnick, using a 27-gauge needle for placing the inoculum in the lumbar enlargement with multiple changes in position during the course of inoculation, obtained a much higher incidence of paralytic manifestations than I did by my method. It is not yet clear whether it is the very thin needle employed in this method or the placement of the inoculum in multiple positions to provide continuous muscular contractions during the period of inoculation, or both, that are important in yielding the more extensive involvement. In at least one test with a 27-gauge needle, Kanda and Melnick (1958) obtained the same results that I did in tests on the type 1 vaccine despite the fact that some of the monkeys received cortisone. A study of the sections of the lumbar cord of monkeys inoculated with the 27-gauge needle and distribution of the inoculum in multiple sites shows that the inoculum is distributed rather widely over many levels of the lumbar cord, and that the retention of most of the inoculum within the spinal cord leads to extensive mechanical disintegration of the grey matter that is associated with a marked inflammatory response.

This method of inoculation is associated with a high incidence of traumatic weakness within 24 hours after inoculation, and the subsequent non-specific inflammatory response and destruction of grey matter can occasionally also lead to further extension of paralysis, for I have seen sections on monkeys with delayed complete paralysis of an extremity in which the non-specific destructive lesions at multiple levels of the cord were obviously responsible for the clinical manifestations. The non-specific destruction of neurones by this method is usually so extensive at multiple levels of the cord that the additional specific viral effect on the contiguous neurones can be sufficient to produce clinical manifestations which would not have occurred as a result of the limited virus activity. Here also, instead of measuring the capacity of the virus to spread to multiple levels of the cord from initially infected cells, the virus is immediately placed at multiple levels of the cord. While this method has obvious advantages for the detection of trace amounts of virus in a killed-virus vaccine, and would also be ideal for testing viruses that are completely devoid of neurotropism in monkeys, it does not appear to be suitable for measuring gradations of activity of strains

that still possess a limited capacity for multiplication and spread from one set of neurones to another.

I have gone to such great length in describing the procedures used in the quantitative estimation of "cerebral" and "spinal" neurotropism, and the factors that must be considered in the interpretation of the results, because it is of the utmost importance for the comparison of results obtained by different investigators as well as for the ultimate standardization and assay of vaccines that may be prepared for human use.

Neurotropism of Viruses Excreted by Vaccinated Individuals and After Multiplication in Contacts

Studies on the neurotropism of virus excreted by individuals after ingestion of attenuated strains of poliovirus have been an integral part of the investigation of every strain, and the results have been reported in a number of previous communications (Sabin, 1955b, 1956a, 1957a, 1957b). It has now been clearly established that when virus cultures, as homogeneous as can be obtained from progeny of single particles by the method of triple-plaque purification, and stable when propagated under specified conditions in monkey kidney tissue cultures, undergo multiplication in the alimentary tract it is possible to show changes in the resulting viral population (Sabin, 1957b). The detection of these changes depends on the use of various selective media. Thus, in testing for virus of increased neurotropism the nervous system of the monkey is the selective medium. If a population of 10 million to 100 million virus particles contains 1,000 particles of greater neurotropism, the nervous system of the monkey can detect their presence, in the same manner as a penicillin-containing medium can detect a small number of penicillin-resistant bacteria among a very large population of penicillin-susceptible organisms—in the first instance it does not mean that the entire viral population has increased neurotropism any more than in the second instance it means that the entire bacterial population is penicillin-resistant.

In the search for virus of increased neurotropism in the stools of vaccinated persons it is important, therefore, on the one hand, to prepare the cultures for monkey inoculation with maximal inocula of virus, and, on the other, to avoid a change in the virus by the method used for cultivation. I have previously reported that the type 2 and type 3 viruses that I selected for incorporation in the vaccine yield 10 to 20 times less virus per cell in cultures with reduced concentrations of bicarbonate than virulent strains, and that even at optimum concentrations of bicarbonate (0.22%) the yield of virus per cell is two to four times higher with the more virulent virus (Sabin 1956a, 1957a). Accordingly it is possible, especially with small inocula of virus, to obtain a viral population in the culture fluid that is not representative of that which existed in the alimentary tract, if the optimum medium is not employed. In my early studies with the plaque-purified type 2 virus excreted in chimpanzee stools, I observed just such an event in which the same stool yielded virus of greatly increased spinal neurotropism when it was grown in monkey kidney cultures which had used up much of their bicarbonate, and no change in neurotropism when it was cultivated in tubes containing fresh medium with a bicarbonate concentration of 0.22%.

Other ingredients in the medium (Li and Jahnes, 1958), including serum (Sabin, unpublished), might also

exert a selective influence in favour of virus of increased neurotropism, and for this reason I have used only monkey kidney tissue cultures maintained in serum-free 0.5% lactalbumin hydrolysate, neutralized with NaOH, prior to dilution in Earle's solution containing 0.22% of NaHCO₃. It may be noted here that Clarke *et al.* (1958) cultured their stools in a poorly buffered medium containing only about 0.1% of NaHCO₃ and 2% calf serum, and that the one stool specimen which yielded virus of specially increased "cerebral" neurotropism (No. 1203—14-day) had only a trace of virus (10^{2.2} TCD₅₀/g.); this means that multiple generations of virus were required in culture with reproduction ultimately occurring at reduced pH and concentrations of NaHCO₃, circumstances that are especially conducive to the selection of more neurotropic virus. One cannot predict what the results might have been if the same stool specimen had been cultivated under more optimum conditions, but the desirability of repeating such tests can hardly be sufficiently stressed.

One of the important questions in this connexion is whether the human alimentary tract may also preferentially select poliovirus of greater neurotropism. At first glance this might appear to be the case because (a) this phenomenon does not occur when the same strains are cultivated in large lots in monkey kidney tissue cultures, and (b) such altered virus is more likely to be encountered in the alimentary tract after it has multiplied for many generations, than in the stools obtained during the first few days after ingestion. The available evidence, however, indicates that the appearance of more neurotropic virus in the human alimentary tract is a chance occurrence. If such altered virus had a selective advantage, one would expect it ultimately to replace the original virus that was fed, and this probably occurs when the original virus has a markedly diminished capacity for multiplication in the alimentary tract, as is the case with the mouse-adapted type 2, TN (Dick and Dane, 1957) and the rodent and chick-embryo-adapted MEF₁ (da Silva *et al.*, 1957) strains.

Several years ago I demonstrated that passage of a type 2 virus in mice selected virus particles with a markedly diminished capacity for multiplication in the alimentary tract (Sabin, 1955a). However, the highly attenuated strains that I selected multiply as extensively in the human alimentary tract as do highly neurotropic viruses, as determined by the amount of virus excreted per gramme of stool in comparison with that found in patients with the naturally acquired paralytic disease (Sabin, 1958). I have previously noted (Sabin, 1957a, 1957b) that when multiplication of virus continues over a long period of time in the human intestinal tract, virus of greater neurotropism that was found to be present earlier could not be detected later, and this has also been observed by Verlinde *et al.* (1958) in children who were fed the type 3 vaccine, and by myself in children who ingested the type 2 and type 3 vaccines. When these viruses are grown in large lots in monkey kidney cultures, there are at most only a few generations of viral multiplication, and any mutants that might arise have little opportunity to find cells that have not already been infected by the original virus. The human alimentary tract, however, provides a very large surface, and mutant clones have a better opportunity to find contiguous or distant uninfected cells. The most plausible explanation for the failure of such mutant clones to survive is that they still have to compete with

much larger populations of the original virus possessing a good capacity for multiplication in the intestinal tract.

Furthermore, if the alimentary tract preferentially selected virus of greater neurotropism one would expect that after millions of years of coexistence with man the naturally occurring polioviruses would all be highly neurotropic, and yet actual tests on polioviruses recovered from the stools of healthy children have shown a wide spectrum of neurotropism with many of the wild strains in the attenuated range (Sabin, 1957b). There is indeed reason to suspect that one of the reasons that epidemics come to an end and then often fail to recur for many years in the same community is not dependent merely on depletion of susceptibles, but that the virus changes on continued multiplication in the alimentary tract. This may be one of the reasons that viruses of mixed characteristics and diminished "cerebral" neurotropism can occasionally be recovered from the stools of paralytic patients, but, in a series of tests that I have carried out, rarely if at all from the spinal cord (Sabin, 1956a).

The observation recorded by Dick and Dane (1958), that the virus recovered from the stools of a healthy contact of a paralytic patient yielded the pattern of a mixed population containing attenuated virus on intracerebral inoculation in monkeys, while the stools of the paralysed patient had distinctly more neurotropic virus, may indeed be an example of this phenomenon. It is also quite possible that, in addition to high neurotropism, a poliovirus may have to possess certain other properties, such as a high capacity for multiplication in extraneural tissues other than the alimentary tract, in order for it to be able to invade the nervous system in sufficient concentration to produce clinical manifestations (Sabin, 1956b). It is noteworthy that most of the naturally occurring type 2 and type 3 viruses are as highly neurotropic as the type 1 viruses, and yet almost all epidemics and about 85% of all paralytic cases are caused by the type 1 virus.

I have previously reported that the three strains that I selected for inclusion in the vaccine to be tested on a progressively larger scale in human beings yielded different results in tests for the presence of virus with increased neurotropism in the stools obtained at different times after ingestion (Sabin, 1957b). Although additional studies are still in progress, I should now like to summarize the additional observations that have been made on these strains since the last report in July, 1957. In all of these tests I have concentrated on stool specimens obtained about a week after ingestion, because the maximum amount of virus is usually excreted at that time and accordingly represents the period when virus is most likely to be transmitted to others, and on the last positive stools to obtain a measure of the subsequent appearance of virus with greater neurotropism.

Type 1 Strain (L Sc, 2 ab)

The previous tests (Sabin, 1957b) for "cerebral" neurotropism on 6- to 7-day stool cultures from 20 adult volunteers and 14- to 28-day stool cultures from eight volunteers, indicated a very high degree of attenuation—only 4 out of 120 monkeys inoculated with very large doses of virus exhibited any paralytic manifestations, and at least one of these was clearly the result of a non-cytopathogenic mutant arising in the monkey. The results of intracerebral tests in 40 monkeys on the stool

TABLE II.—*Intracerebral Neurotropism of Cultures of Type 1 Poliovirus Excreted by Triple-negative Children at Different Times After Ingestion of Attenuated L Sc, 2 ab Strain*

Name	Age in Years	Days after Ingestion	TCD ₅₀ Inoculated Log 10	Result	
				Clinical*	Histological†
A. S.	5	7	7.4	0, 0, 0, 0	0, 0, 0, +
		20	7.1	0, 0, 0, 15	0, +, +, +
D. S.	7	7	7.4	0, 0, 0, 0	0, 0, 0, 0
		20	7.4	0, 0, 0, 0	0, 0, 0, 0
S. F.	5	10	6.9	0, 0, 0, 0	0, 0, 0, 0
		21	7.7	0, 0, 0, 0	0, 0, 0, 0
P. F.	9	7	7.1	0, 0, 0, 0	0, 0, 0, 0
		19	7.1	0, 0, 12, 18	0, ±
K. F.	11	5	7.7	0, 0, 0, 0	0, 0, 0, +
		17	7.1	0, 0, 0, 0	0, 0, 0, 0

The indicated dose of virus was administered in a volume of 0.5 ml. into the right thalamic region of cynomolgus monkeys weighing about 6 lb. (2.7 kg.).

* Clinical:—15=Monkey exhibited partial paralysis of lower extremities 15 days after inoculation. 0=No paralysis observed. 18=Weakness of right upper extremity 18 days after inoculation, which by 21 days progressed to partial paralysis; no cytopathogenic virus was found in the spinal cord (various dilutions tested to avoid zone), but focal poliomyelitis lesions limited to one side of the cervical and upper thoracic cord were present.

† Histological examination involved 60 to 80 levels of spinal cord of the clinically negative monkeys. 0=No poliomyelitis lesions. ±=One old, focal neuronal lesion involving only one level of 84 examined. +=Focal lesions in many levels but little or no loss of neurones. ++=More extensive than + but many levels entirely negative.

cultures from five triple-negative children, shown in Table II, are of a similar nature, with only three monkeys showing paralytic manifestations—one of them again clearly due to a non-cytopathogenic mutant originating in the monkey. The occurrence of limited, focal neuronal lesions in only 5 of the 37 monkeys that remained clinically well, on the one hand, indicates the "cerebral" innocuity of the recovered virus, and on the other points to the possible presence of at least some virus with an increased capacity for multiplication in the cerebral neurones, for no such lesions have been found in any of the monkeys after intracerebral inoculation of the original virus.

The tests for "spinal" neurotropism on the virus in late stools from eight adult volunteers all showed some increased activity. Although the spinal titrations have not yet been completed on all the stool cultures from the five triple-negative children, a distinct increase in paralytogenic activity even with doses in the range of 10^4 TCD₅₀ and less were found in at least three. Studies now in progress on 10 additional children also show an increase in "spinal" neurotropism. I was unsuccessful in obtaining spread of the type 1 virus in one institutional study where about 90% of the children were naturally immune to this type and therefore had no stools of contacts to study. Material on contacts made available by Drs. John Fox and Henry Gelfand from their family studies in New Orleans is now under investigation.

Verlinde *et al.* (1958) tested for "spinal" neurotropism 19 stool cultures from 12 children and one adult, who received an aliquot of the same large lot of the type 1 vaccine; these included seven cultures of stools obtained 14 to 21 days after ingestion. Each culture was inoculated undiluted and diluted 1:10 into each of two cynomolgus monkeys, and the incidence of paralytic manifestations was less than I obtained with the original virus (Table I). Among 58 monkeys inoculated with 10^6 to $10^{8.5}$ TCD₅₀, only 4 exhibited any paralytic manifestations, and among 18 monkeys inoculated with $10^{4.8}$ to $10^{5.8}$ TCD₅₀, none was paralysed. They also obtained no evidence of paralysis in 10 monkeys inoculated with five original stool suspensions in doses of 10^2 to $10^{1.3}$ TCD₅₀. In my

preceding discussion on methods of inoculation I proposed a possible explanation for the difference in the results obtained by Verlinde and myself. Although the data of Verlinde *et al.* (1958) cannot be used as a measure of increase in neurotropism of excreted virus, they nevertheless show that the viruses they recovered were distinctly attenuated.

Smorodintsev *et al.* (1958) reported a most interesting series of studies on children who were fed type 1 virus prepared from the terminal dilution purified L Sc strain that I supplied them. This is the strain that I found to be inhomogeneous upon examination of the progeny from different plaques (Sabin, 1956a), and from which I selected the optimum L Sc, 2 ab triply purified plaques for the type 1 vaccine. Smorodintsev *et al.* carried out their studies in institutions in which 95% of the children were triple-negative, and observed extensive spread of this virus. While they observed a slight increase in neurotropism of the excreted virus, it was no greater after an estimated four to five natural human passages than it was in the stools of the children who received the original culture fluid. They, furthermore, carried out eight serial passages of the virus in children—the stools of several children obtained two to four weeks after feeding were cultured in monkey kidney, tested in monkeys, and then fed to another group of triple-negative children for eight consecutive passages—without observing any increase in neurotropism beyond that found in the stools excreted by the children who received the original culture fluid.

Horstmann *et al.* (1957a), working with an aliquot of the terminal dilution purified L Sc strain that I supplied to them, reported that tests in monkeys on the last positive stool specimens representing first and second human passage materials “showed very slight increases in virulence” in only two of nine specimens tested.

Type 2 Strain (P 712, Ch, 2 ab)

The previous tests (Sabin, 1957b) for “cerebral” neurotropism on 5- to 10-day stool cultures from 12 adult volunteers and 21- to 61-day stool cultures from five volunteers, indicated a high degree of attenuation—only 5 out of 81 monkeys inoculated with very large doses exhibiting any paralytic manifestations, all five occurring with the 7- and 21-day stools of one of the 12 volunteers. The results of intracerebral tests in 66 monkeys on 12 7- to 56-day stool cultures obtained from the same group of five triple-negative children that were fed the type 1 and type 3 vaccines prior to receiving the type 2 virus are of a similar nature (Table III).

In this study the initial screening tests were purposely carried out with a 1-ml. inoculum, and it is noteworthy that in each instance in which some of the monkeys exhibited paralysis (A. S., 36-day; P. F., 36-day; K. F., 10-day) and the test was repeated, giving the same dose of virus in a 0.5-ml. inoculum, no paralysis was observed. It is also noteworthy that in three of the paralysed monkeys tissue-culture tests on the spinal cord yielded a zone phenomenon indicating the presence of both non-cytopathogenic and cytopathogenic virus, and in one paralysed monkey no cytopathogenic virus was found in any dilution of the spinal cord despite the presence of typical polio lesions—findings indicative of the effects of mutants arising within the nervous system of the monkeys. Another finding of interest is that in the instances in which some paralytic monkeys were

TABLE III.—*Intracerebral Neurotropism of Cultures of Type 2 Poliovirus Excreted by Initially Triple-negative Children at Different Times After Ingestion of Attenuated P 712, Ch, 2 ab Strain Administered After Previous Feedings of Types 1 and 3 Viruses*

Name	Days after Ingestion	Result after Inoculation of Indicated Amounts (Log 10)							
		Dose Paral.		Dose Paral.		Dose Paral.		Dose Paral.	
A. S.	7	8.4	0, 0	6.4	0, 0				
	36	8.0	0, 0	6.0	0, 13				
	56	7.2	0, 0	5.5	0, 0	5.0	0, 0	4.5	0, 0
D. S.	9	7.7	0, 0	5.7	0, 0				
	35	8.2	0, 0	6.2	0, 0				
S. F.	8	7.6	0, 0	5.6	0, 0				
	35	7.7	0, 12*	5.7	0, 0				
P. F.	10			6.7	0, 0	4.7	0, 0		
	36	8.2	0, 8	6.2	0, 12*				
	55	7.1	0, 0	5.4	0, 0	5.2	0, 0	4.4	0, 0
K. F.	10	8.0	0, 0	6.0	17*, 17†				
	20	8.4	0, 13 tr.	6.4	0, 0	5.0	0, 0	4.0	0, 0

The initial inoculations of the undiluted and 10⁻² dilutions of the culture fluids (except the 55- and 56-day specimens) were all in a volume of 1 ml. given entirely into the right thalamic region. In the repeat tests the same dose of virus was given in a volume of 0.5 ml.; the results for 1 ml. are given in the numerator and those for 0.5 ml. in the denominator when the same dose was given in different volumes.

tr = Paralysis slight and transitory.

* Zone phenomenon in test for virus in spinal cord, indicating presence of both non-cytopathogenic and cytopathogenic virus.

† No cytopathogenic virus found in any dilution of spinal cord suspension.

found in the tests on the 36-day stools (A. S. and P. F.) no paralysis was found in any of the monkeys inoculated with the 55- or 56-day stools from the same children. This failure of more neurotropic virus to accumulate or persist in the alimentary tract was confirmed by tests for “spinal” neurotropism on the same specimens.

The previous tests for “spinal” neurotropism showed a definite increase in the “late” stools of at least two of the four volunteers tested, and the tests on the stools of the five children showed a similar, sometimes transitory, increase in four. Here again it is noteworthy that Verlinde *et al.* (1958), who tested 11 stool cultures from seven children after ingestion of an aliquot from the same type 2 vaccine, found no evidence of “spinal” neurotropism. Their tests included cultures on five stools obtained 13 to 23 days after ingestion; only one of 26 monkeys receiving 10⁶ to 10^{7.7} TCD₅₀ of virus exhibited paralysis (the one positive monkey received 25 million TCD₅₀), and none of 12 inoculated with 10⁵ to 10^{5.8} TCD₅₀, and none of six inoculated with 10^{4.3} to 10^{4.5} TCD₅₀. Again, while their results cannot be used as a measure of increase in neurotropism, because of the method they used, they nevertheless point to the attenuated characteristics of the excreted virus.

Smorodintsev *et al.* (1958) carried out studies with type 2 virus similar to those described for type 1. The virus they used was prepared from the “P 712, 10 ab₁” strain that I sent them. This is the triply purified plaque progeny from which I selected the P 712, Ch, 2 ab strain used for preparation of the type 2 vaccine (Sabin, 1956a, 1957a, 1957b). In my preliminary tests with the P 712, 10 ab₁ strain in chimpanzees, I found that some excreted virus of increased “spinal” neurotropism and one did not. I therefore used the stool culture of the one chimpanzee which yielded negative results on spinal inoculation, for

further analysis of single-plaque progeny and selected the P 712, Ch, 2 ab progeny, but there is probably no difference between it and the P 712, 10 ab₁ strain. Smorodintsev *et al.* (1958) found no evidence of greater neurotropism of the virus in the stools of contacts than in those who received the original culture fluid. Furthermore, during the course of five experimental consecutive passages in triple-negative children they observed a partial increase in both "cerebral" and "spinal" neurotropism in the stools of the fourth human passage, which was no longer found in the stools of those who received it for the fifth human passage.

Type 3 Strain (Leon 12 a,b)

The previous tests (Sabin, 1957b) for "cerebral" neurotropism on 11 6- to 7-day and on 11 20- to 61-day stool cultures from adult volunteers in 137 monkeys indicated a greater increase in neurotropism of the virus in the stools of 2 of the 12 volunteers studied than was observed with the type 1 and type 2 viruses. Here, also, there was evidence that the paralysis in a few monkeys was associated with mutants arising in the nervous system of the monkeys, because no cytopathogenic virus could be recovered from the spinal cord. The results of intracerebral tests in 99 monkeys on 11 stool cultures and five original stools obtained 6 to 21 days from the same group of five triple-negative children who received the type 3 vaccine 21 days after the type 1 are shown in Table IV. Here also the initial screening tests were purposely carried out with a 1-ml. inoculum, and in several instances different results were obtained when the same dose was inoculated in 0.5 ml. The greater "cerebral" neurotropism is also evident in these tests, but it is noteworthy that in all instances the results are indicative of a mixed population of virus particles in that some monkeys inoculated with larger amounts failed to develop paralysis while others receiving smaller amounts did. Paralysis also appeared in 3 of the 19 monkeys inoculated with the original stools, but the zone phenomena found in the tissue-culture tests on the spinal cords of two and the prolonged incubation period in the third suggest the possibility that the paralysis may have resulted from mutants arising during the course of multiplication of

the virus in the nervous system of the monkeys. It is again noteworthy that in child S. F., the 20-day stool cultures gave completely negative results (with 1-ml. inocula) while the 6-day and 13-day stools obviously contained some virus of greater neurotropism.

During the course of a recent study in association with Krugman, Giles, Barnes, and Wang (unpublished), in which the type 3 vaccine was fed to children who had previously had four doses of Salk vaccine, stools were also collected from a number of contacts who lived with them in the same institution under conditions particularly conducive to faecal spread. Serological tests on these children over a period of 15 months prior to administration of the oral vaccine gave no indication of spontaneous infection with polioviruses, although there was ample evidence that other enteroviruses of one kind or another were in almost constant circulation. Type 3 poliovirus appeared in three of six contacts whose stools were examined at frequent intervals. The time of appearance of the virus in their stools shown in Table V suggests that at least one of the children may have acquired his infection after more than one passage in the intestinal tract of the others. The results of the preliminary tests for cerebral neurotropism with 1-ml. inocula show the attenuated characteristics of the virus that has multiplied for many additional generations in their intestinal tracts.

In another unit of this institution a type 1 test was in progress at the same time. These children had no direct contact with those who received the type 3 vaccine, but about six weeks after the beginning of the test type 3 virus was isolated from eight children whose stools were being tested at frequent intervals. The only indirect contact between the two units may have occurred through the medium of night attendants who administered intramuscular injections of medication in the buttocks. Although no virus carriers were found among the five attendants four weeks after the first appearance of the type 3 virus in the second unit, the possibility, nevertheless, exists that they may have introduced it, although it is also possible that a "wild" type 3 virus was introduced from outside. The preliminary tests on the stool cultures of these children, shown in Table V, show that the virus was of an attenuated character.

TABLE IV.—*Intracerebral Neurotropism of Some Original Stool Suspensions and Stool Cultures of Type 3 Poliovirus Excreted by Triple-negative Children at Different Times after Ingestion of Leon 12 a,b Strain Administered after Previous Feeding of Type 1 Virus*

Name	Days after Ingestion	Stool Cultures—Dose Log 10						Original Stool	
		Dose	Paral.	Dose	Paral.	Dose	Paral.	Dose	Paral.
A. S.	15 21	8.4	0, 5	6.4 6.7	0, 0 0, 0		4.7 0, 0		
D. S.	9 21	8.2 9, 9 7.4 9, 13		6.2 6, 13 tr. 0, 10		5.2 13*, 14 5.4 8, 13 0, 0	4.2 0, 16 4.4 0, 12*	3.2 0, 12 3.4 0, 0 2.4 0, 0	3.4 0, 0, 16* 2.4 0, 0, 0, 12*
S. F.	6 13 20	8.2 0, 15 7.4 slight 7.4 17, 0 0, 0		6.2 0, 11 0, 16		5.2 0, 0 5.7 10, 15 5.4 0, 0	4.2 0, 0 4.7 0, 0	3.7 9, 16	4.4 0, 0, 0, 24 4.9 0, 0, 0, 0
P. F.	6 14	8.7 0, 0 8.7 10, 13 tr.		6.7 0, 16 0, 0 0, 0		5.7 0, 25	4.7 0, 0		
K. F.	6 20	8.4 0, 12 7.4 17, 20 tr. tr.		6.4 0, 0		5.4 0, 20* 0, 13	4.4 0, 22		4.0 0, 0, 0, 0

See notes at foot of Table III.

Verlinde *et al.* (1958) tested 24 stool cultures obtained from 14 children 4 to 44 days after ingestion of aliquots of the same large lot of type 3 vaccine that I used in my studies. These cultures, 14 of them representing 15- to 44-day stools from nine children, were tested intraspinally in 96 cynomolgus monkeys and intracerebrally in 22 monkeys. Seventeen specimens from 12 children yielded negative results—only 2 out of 54 monkeys inoculated intraspinally with 1 million to 100 million TCD₅₀ exhibiting any paralysis. In two children there was definite evidence of a transitory presence of more neurotropic virus by both the spinal and the intracerebral methods of testing: by the latter method, only the monkeys inoculated with amounts in excess of 1 million TCD₅₀ exhibited paralysis. In both children the last stool specimens, in one instance obtained 21 days and in the other 33 days after ingestion, gave completely negative results in the monkeys that received large doses of virus by their method of spinal inoculation. Although the methods of testing were different, it is nevertheless evident that in all instances the excreted viruses were of an attenuated character.

Clarke *et al.* (1958) reported intracerebral tests in monkeys on 10 stool cultures obtained from five children 1 to 31 days after feeding an aliquot of the same large lot of type 3 vaccine. It has already been pointed out that the highly traumatic technique of intracerebral inoculation that they used (0.5 ml. into each thalamic region) is not suitable for an accurate measure of "cerebral" neurotropism, and that the culture medium they used for enriching the amount of virus in the stools was conducive to increasing the proportion of more neurotropic virus that might have been present there. Nevertheless, the results that they reported were (with one possible exception) not significantly different from those I had previously reported as indicating that a variable proportion of the

virus excreted after ingestion of the type 3 vaccine is more neurotropic than that which was fed. Some of the monkeys that they recorded as paralytic, particularly those with relatively short incubation periods after inoculation of about 10 million to 140 million TCD₅₀ of virus, may represent animals in which the virus invaded the spinal cord *directly* as a result of the traumatic inoculation, while others were apparently recorded as paralytic when the cause of their paralysis was not the poliovirus found in the spinal cord.

Thus, according to a personal communication from Dr. Goffe, a monkey recorded as having its onset of paralysis six days after inoculation of 890,000 TCD₅₀ of the 7-day stool culture of case 1201, actually died of bacterial meningitis and exhibited only minimal polio lesions. It has already been pointed out that rather extensive specific poliomyelitic lesions can be found in monkeys without any clinical evidence of paralysis, and only the loss of a great many neurones in multiple levels of the cervical or lumbar cord constitutes histological confirmation that paresis or ill-defined paralysis, especially when it is not clearly flaccid, has been caused by the action of poliovirus. There is also evidence that paralysis in some of their monkeys was associated with non-cytopathogenic mutants which must have arisen in the nervous system of the monkeys, for in their cases 1205 and 1308 they failed to recover virus in tissue culture from three of the five monkeys recorded as being paralytic.

Dr. Goffe also wrote me that, in 5 of the 16 cords from which virus was recovered, a cytopathogenic effect was demonstrable only in the second passage, suggesting that the original material probably contained interfering non-cytopathogenic virus. The virus excreted by four of the five children was of a distinctly attenuated character, and only the 14-day stool culture of case 1203 yielded results comparable to those I found in child D. S. (Table IV). The need for repeating the tests on

TABLE V.—Preliminary Tests for Intracerebral Neurotropism of Type 3 Virus Grown from Stools of Children Having Direct or Indirect "Contact" with Children who were Fed Type 3 Vaccine

Type of Contact	Name	Days after Feeding Vaccine to Others Stools of Contacts were:			TCD ₅₀ Inoculated Log 10	Clinical Result
		Negative for Type 3 Virus	Positive for Type 3 Virus	Used for Monkey Test		
Direct 2 or more human passages of virus	Hel.	0, 2, 4, 10, 27, 41, 52, 59	6, 14, 20	20	7.7 5.7	0, 0 21*
	Hir.	0, 2, 4, 6, 9, 20, 27, 41, 50, 55	13	13	8.5 6.5	0, 0 0, 0
	Fis.	0, 2, 4, 6, 9, 20, 28, 52, 55	42	42	7.7 5.7	0, 14 0, 18
Indirect 3 or more human passages of virus or Introduction of "wild" virus into unit	Bos.	0, 2, 4, 6, 9, 13, 20, 28, 52, 55	41	41	8.2 6.2	0, 7 0
	Dil.	0, 2, 4, 6, 9, 13, 20, 27, 50, 55	41	41	7.4 5.4	7, 10 0, 8 transit
	Ros.	0, 3, 5, 6, 10, 11, 14, 20, 27, 55	41, 50	41	7.2 5.2	6, 7 0, 7
	Sch.	0, 2, 4, 6, 10, 13, 21, 27, 55	41, 50	50	7.9 5.9	4, 5 0, 16
	Hau.	0, 2, 4, 6, 9, 13, 20, 27, 56	41, 51	51	7.2 5.2	0, 17 0, 6
	Kee.	0, 2, 4, 6, 9, 13, 20, 27	41, 50, 55	55	6.9 4.9	0, 9 0, 13
	Ort.	0, 2, 4, 6, 9, 13, 20, 27	41, 50, 55	55	7.4 5.4	0, 12 transit 0, 21†
	Lev.	0, 3, 5, 7, 10, 14, 28, 42, 49	50	50	7.2 5.2	6, 8 0, 1

* Only trace of cytopathogenic virus recovered after long delay despite presence of acute polio lesions in spinal cord.

† Marked zone phenomenon in tissue culture indicative of presence of non-cytopathogenic interfering virus in spinal cord.

Note.—All monkeys in this test were inoculated with 1 ml. into right thalamic region.

such stool specimens with cultures containing optimum medium, to obtain a better estimate of the characteristics of the virus present in the original stool, were discussed earlier.

Smorodintsev *et al.* (1958) carried out studies with the type 3 virus similar to those described for the types 1 and 2 viruses, and obtained similar results. The virus they used was prepared from the triply purified, Leon 14 ab, plaque progeny that I sent them. The Leon 12 a₁b plaque progeny, which I finally selected for inclusion in the vaccine, and the Leon 14 ab plaque progeny, were both derived from the same attenuated, terminal-dilution-purified "Leon" virus (Sabin, 1956a, 1957a, 1957b), and were the only plaque progeny which on repeated tests failed to produce paralysis in any of the monkeys inoculated intraspinally (by the method I described) with the largest doses of virus. In preliminary feeding tests in chimpanzees, virus of increased neurotropism was excreted by those receiving the 14 ab strain but not by those receiving the 12 a₁b strain (Sabin, 1957b), and that was the reason why I selected the Leon 12 a₁b strain for the type 3 vaccine. Smorodintsev *et al.* (1958) reported that highly attenuated virus was excreted by the contacts as well as by the children who received the original culture fluid. Furthermore, during the course of six experimental consecutive passages in triple-negative children, they found that a partial increase in both "cerebral" and "spinal" neurotropism, which was observed in the stool cultures of the fifth human passage, was not evident in the sixth passage.

Other Methods for Testing Variations in Characteristics of Excreted Virus

On the basis of all information available to me there is thus far no *in vitro* method that can be substituted for monkeys in measuring the *degree* of neurotropism of a poliovirus. I have previously pointed out that the type 2 and type 3, but not type 1, highly attenuated strains produced much smaller plaques under agar overlays with full (0.22%) concentrations of NaHCO₃ than their highly neurotropic parents (Sabin, 1957a), but I found that the highly attenuated type 3 Leon 12 a₁b virus, which showed no increase in neurotropism after two weeks of multiplication in the posterior pharyngeal wall of chimpanzees, nevertheless produced distinctly larger plaques than the virus that was fed.

The capacity to produce plaques under agar overlays containing a reduced (0.05%) concentration of NaHCO₃ (the so-called "d" character) has been found lacking in all highly attenuated strains (Vogt *et al.*, 1957), but I have already reported that the progeny of four plaques, especially selected by Dulbecco and Vogt from the Leon 12 a₁b and Leon 14 ab triple-plaque purified strains by picking late mutants on cultures with "acid" agar overlays, exhibited no change in spinal neurotropism for monkeys, although they possessed a 100 to 1,000 times greater capacity for producing plaques under "acid" overlay (Sabin, 1957a).

Hsiung and Melnick (1958), in a comprehensive study on the relation of the "d" character to neurotropism in naturally occurring and experimentally attenuated strains of poliovirus, as well as in polioviruses excreted after the feeding of attenuated strains, found no evidence of marked "cerebral" neurotropism in many "d+" strains exhibiting a high efficiency of plaque formation under overlays with reduced concentrations

of NaHCO₃. They also quoted unpublished studies of Horstmann and Maas, which indicated (a) that misleading information regarding the "d" character of poliovirus in stools may be obtained if the specimen is cultured in a low-bicarbonate instead of a high-bicarbonate medium; and (b) that virus exhibiting a high efficiency of plaque formation under low-bicarbonate agar overlay (d+ character) can be distinctly attenuated when tested for neurotropism in monkeys.

Recently Kanda and Melnick (1958) reported that neurotropically virulent and attenuated polioviruses grew differently on primary monkey-kidney tissue cultures and on a stable line of monkey-kidney cells (the "MS" line)—the attenuated strains yielding 1/10,000 the amount of virus and distinctly smaller plaques in the "MS" cells. A total of 110 stool and stool-culture specimens from 40 volunteers obtained at different times after ingestion of portions of the large lots of the type 1, 2, or 3 vaccine, which I had previously tested for neurotropism (Sabin, 1957b), were tested in Dr. Melnick's laboratory for the so-called "MS" character—that is, the capacity to grow equally well on the "MS" line of cells as on primary monkey-kidney cells, a property possessed by the virulent polioviruses.

It is noteworthy that the virus present in 18 original stool extracts and in 18 stool cultures from 10 volunteers, obtained 7 to 61 days after ingestion of the type 3 vaccine, and in 13 original stool extracts and in 16 stool cultures from 10 volunteers, obtained 7 to 61 days after ingestion of the type 2 vaccine, in all instances lacked the "MS" character of the virulent polioviruses, despite the fact that the monkey tests on the same specimens showed a distinct increase in neurotropism in at least one of the type 2 volunteers and two of the type 3 volunteers. The tests on 19 original stool extracts, obtained from as many volunteers six to seven days after ingestion of the type 1 vaccine, indicated a partial "MS" character in six, which in five instances was not detected in the cultures prepared from them. The 28-day culture from the one volunteer, whose seven-day original stool and stool culture were "MS" positive, as well as 24 other stool cultures, obtained from 19 other volunteers 6 to 28 days after ingestion of the type 1 vaccine, all were "MS"-negative—that is, possessed the characteristics of the neurotropically attenuated polioviruses. The one "MS"-positive stool culture had no greater "spinal" neurotropism in monkeys than the "MS"-negative stool cultures.

Persistence of Antibody and Resistance of the Alimentary Tract to Infection

I have previously reported that the antibody response resulting from multiplication of attenuated polioviruses in the alimentary tract varies with the individual, the age of the person, and the strain and type of virus fed (Sabin, 1956a, 1958). Accordingly, observations based on a single strain, particularly one that has a limited initial capacity for multiplication in the human alimentary tract, cannot properly be used for generalizations about the effects of attenuated polioviruses (Dick and Dane, 1958; Dane *et al.*, 1958). Furthermore, the statement occasionally encountered in the literature, that natural infection results in the development of neutralizing antibodies of high titre

which persist without significant change in level, is now known to be incorrect.

The recent studies of Lennette and Schmidt (1957) on patients with clinical poliomyelitis have shown that some respond with high titres and others with low, and that in most individuals the titre drops from fourfold to twentyfold during the course of a year or two following infection. Le Bouvier (1957), in a study of antibody levels in persons of different age in different population groups, also obtained evidence of a decline in levels over the years, even in unsanitary areas where polioviruses are extensively disseminated, suggesting that reinfection with the consequent boost in the level of antibody does not occur in such populations after a certain period of years. In my studies on adult human volunteers I have repeatedly encountered individuals with minimal or no demonstrable neutralizing antibodies for one or more types of poliovirus who exhibited complete resistance to infection of the alimentary tract with large doses of attenuated poliovirus—that is, the virus not only failed to multiply but there was also no antibody response (Sabin, 1956a, 1957b, 1958).

TABLE VI.—Persistence of Type 1 Antibody Resulting from Feeding Various Attenuated Type 1 Polioviruses Correlated With Resistance of Intestinal Tract to Subsequent Feeding of About 10^6 TCD₅₀ of Virus*

Strain Originally Fed	Name	Interval after Feeding (Months)	Antibody			Resistance of Intestinal Tract at Indicated Time
			pH	Cytopathogenic		
				2-Day	8-Day	
L Sc, 2 ab	A. S.	3	2,048	3,200	3,200	
		9		3,200	3,200	
	D. S.	3	2,048	640	640	
		9		2,000	320	
L Sc ... (Terminal dilution progeny)	Moo.	3	8	16	16; 4	Complete*
		16	8	16	6	
	Ree.	3	48	64	48; 20	"
		16	32	48	12	
	And.	3	64	12	0	"
		9	48	4	0	
	Joh.	2	12	16	4	"
		9	24	16	4	
	Klo.	3	192	320	32	"
		9	128	320	32	
Smi.	3	128	200	32	Partial†	
	9	64	32	32		
" P 2149 "	Col.	3	384	320	320	Complete
		16	1,024	640	320	
	Cou.	3	384	640	320	
		16	384	640	320	
" P 2226 "	Dam.	3	512	640	320	"
		16	256	320	320	
	Dil.	3	128	256	180; 32	
		16	96	256	48	
Mahoney KP 33	Hac.	3		2,000	640	
		12			2,000	
		30			3,200	
	Har.	3			320	
		12			320	
	Mal.	3			32	
		12			32	
	Tro.	3			4	
		12			20‡	
	Ric.	3			32	
		12			32	
	Car.	3			32	
		6			32	
	Wal.	3			32	
	6			32		

* The challenge virus was the L Sc, 2 ab strain; and five controls, without homotypic antibody, who were fed the same dose at the same time all developed intestinal infection. Complete resistance=No virus detected in stools between 5 and 28 days after ingestion and no rise in antibody.

† Partial resistance=Virus found in stools for first 14 days in smaller than usual amounts associated with distinct rise in antibody. Unlike the other volunteers this man exhibited early interference followed by limited viral multiplication for a period of seven days after the first feeding nine months earlier.

‡ This man received type 2 virus at three months, which was followed by a boost in his type 1 antibody.

Note.—Only A. S. and D. S. are children. The others were all young adults, living away from children, in an environment where no spontaneous spread of polioviruses was detected.

TABLE VII.—Persistence of Type 2 Antibody Resulting from Feeding Various Attenuated Type 2 Polioviruses Correlated With Resistance of Intestinal Tract to Subsequent Feeding of About $10^{5.7}$ TCD₅₀ of Virus*

Strain Originally Fed	Name	Interval after Feeding (Months)	Antibody			Resistance of Intestinal Tract at Indicated Time
			pH	Cytopathogenic		
				2-Day	8-Day	
P 712, Ch, 2 ab	A. S.	1-25	256	320	200	
		8		320	32	
	D. S.	1-25	512	320	320	
		8		320	32	
P 712 (Terminal dilution progeny)	Wal.	3	768	320	32	Complete
		8	512	320	200	
	Wai.	3	512	32	32	"
		8	512	32	32	
	Boy.	3	768	320	320	"
		9	1,024	320	64	
	Bes.	3	768	200	32	"
		15	1,024	200	64	
	Die.	3	768	320	200	"
		15	768	200	32	
	Tru.	3	2,048	2,000	200	"
		15	1,536	320	64	
Wat.	3	1,024	320	200	Partial†	
	15	1,024	200	64		
YSK	Pee.	3			200	
K P 51		7			320	
		13			320	
	Dou.	3			200	
		6			200	
	Ros.	3			32	
		6			64	
	Nic.	3			32	
		6			320 + ‡	

* The challenge virus was the P 712, Ch, 2 ab strain; and five controls, without homotypic antibody, who were fed the same dose at the same time all exhibited intestinal infection.

† Partial resistance—Small amounts of virus detected in stools for period of 10 days associated with rise in antibody. It is noteworthy that, unlike the other volunteers, this man was originally fed a mixture of types 1 and 2 polioviruses, and both multiplied simultaneously.

‡ This man received type 3 virus at three months, which was followed by a boost in his type 2 antibody.

The data shown in Tables VI, VII, and VIII summarize the results on persistence of antibody in 19 individuals who had been fed various type 1 strains, 13 individuals fed various type 2 strains, and 10 individuals fed the attenuated type 3 "Leon" virus. The data on tests for resistance of the alimentary tract to reinfection, in relation to the level of antibody at the time of the test, are also shown for 24 of these individuals. The results are given separately for the combined low-avidity and high-avidity antibody, as determined by the pH method and partly also by early (two-day) readings in the cytopathogenic method, and for high-avidity antibody represented by the eight-day reading in the cytopathogenic test (using serum-virus mixtures that are incubated only one hour at room temperature and subsequently undergo a 1:11 dilution in the culture fluid of the tubes used for the test). The titres are the 50% end-points calculated for 0.2 ml. of the original serum; the dilution which the serum-virus mixture undergoes following addition to the test system is not involved in this calculation. With few exceptions, the comparative tests for level of antibody at different times were carried out simultaneously with sera that had been preserved in the frozen state.

While there are certain variations in the levels of high-avidity and low-avidity antibody during the first three months after infection, when the antibody changes in its qualitative characteristics (see Table IX), it is evident that the level of antibody present at three months had undergone little or no significant change in titre during the subsequent periods, varying from 6 to 16 months after feeding, except for the rises in the few individuals who were fed other types of poliovirus in the interim. In the type 2 group (Table VII) the

high-avidity titre showed a drop of threefold to tenfold in six instances, while the low-avidity antibody titre remained unchanged. It is particularly noteworthy that the persistence of antibody was not dependent on the titre—the low levels persisted as well as the high ones. Of special interest is the fact that resistance of the

alimentary tract was in no way related to the level of antibody, and in three instances complete or marked resistance was present in individuals without high-avidity antibody and only minimal levels of low-avidity antibody. It must be remembered that on primary exposure to the virus there was multiplication in the alimentary tract of all of these individuals. These data, taken together with the demonstration that a certain proportion of adults without demonstrable antibody by the usual methods have been found to have resistant alimentary tracts, indicate that the persistent resistance of the alimentary tract to infection with poliovirus is not dependent on persistence of antibody.

Recent studies in association with Dr. Giacomo Messore on large numbers of stools from known susceptible and immune children and adults, prior and subsequent to infection with orally administered attenuated polioviruses, showed that resistance of the alimentary tract is not associated with the presence of any specific antibody or other neutralizing principle in the faeces. The high susceptibility of the intestinal tract in the presence of high levels of neutralizing antibody in the blood resulting from killed-virus vaccine, and the high resistance of the intestinal tract in the absence of readily demonstrable antibody in the blood or faeces, strongly suggest that the resistance is based on specific cellular rather than humoral factors. It also follows from this that intestinal infections that are originally limited in extent by virtue of dose, simultaneous infection with more than one type, interference by other enteric viruses, or inadequate multiplication of an experimentally attenuated virus, may result in only partial resistance without reference to demonstrable antibody—and this is borne out by some of the data in Tables VI, VII, and VIII, as well as by observations on human beings with naturally acquired antibody (Sabin, 1957b; Fox *et al.*, 1957; Horstmann *et al.*, 1957a).

TABLE VIII.—Persistence of Type 3 Antibody Resulting from Feeding Attenuated Type 3 Poliovirus Correlated with Resistance of Intestinal Tract to Subsequent Feeding of About $10^{5.5}$ TCD₅₀ of Same Strain*

Strain Originally Fed	Name	Interval after Feeding (Months)	Antibody			Resistance of Intestinal Tract at Indicated Time
			pH	Cytopathogenic		
				2-Day	8-Day	
Leon 12 a ₁ b	A. S.	2	128	64	50	
		8.5	48	48	16	
	D. S.	2	64	64	12	
		8.5	32	24	24	
Leon (Terminal dilution progeny)	Bye.	3	64	32	6	Complete
		9	32	32	32	
	How.	3	256	320	200	
		9	256	320	200	,,
	Tod.	3	256	320	64	
		9	128	200	32	
	Per.	3	128	64	32	,,
		8.5	128	32	32	
	Tom.	3	128	32	3	
		8.5	128	64	3	,,
	Qui.	3	16	8?	4	
		8.5	8	4?	0	
	Sul.	1	16		20	Marked†
		2	16		3	
		3	16		0?	
			6			2
		12			0?	
Rod.		3			4	
		6			32‡	
		9			32	

* The challenge virus was the Leon 12 a₁b strain; and 13 controls, without hemotypic antibody, who were fed the same dose at the same time all exhibited intestinal infection.

† Marked resistance—Only a trace of virus was detected once in the stools on the 14th day after feeding, and this was associated with a marked rise in antibody.

‡ This man received type 2 virus at three months, which was followed by a boost in his type 3 antibody.

TABLE IX.—Low-avidity and High-avidity Neutralizing Antibody in Young Adults Following Ingestion of only One Type of Vaccine. Variation in Relation to Type of Virus Ingested, Dose, Extent, and Duration of Excretion of Poliovirus in Stools

Type of Vaccine	Dose	Name	Excretion of Virus		Antibody Response at Indicated No. of Days							
			Duration Days	Peak Titre TCD ₅₀ g.-Log 10	Low + High Avidity—pH				High Avidity Only			
					Pre	14	28	61	Pre	14	28	61
1 L Sc, 2 ab	0.01 ml. 4.2 × 10 ⁵ PFU	Bli.	28 + ?	6:2	<4	256	256		0	64	20	
		Hou.	28	5:2	<4	256	4,096	1,024	0	0	320	200
		Mar.	21	5:7	<2	256	64	64	0	32	20	4
		Koc.	21	4:2	<2	1,024	512	256	0	320	320	320
		Gri.	21	3:7	<2	512	512	256	0	64	32	32
		Bur.	14	5:2	<2	2,048	1,024	512	0	320	320	320
		Dav.	14	4:2	<2	2,048	2,048	512	0	320	320	64
		Mil.	14	4:2	<2	2,048	2,048	2,048	0	2,000	320	320
		Lay.	14	3:7	<2	1,024	2,048	1,024	0	200	320	320
		Eil.	10	5:2	<2	1,024	512	256	0	64	200	200
		Kyz.	10	3:7	<2	512	512	256	0	64	32	32
		Oli.	10	4:2	<2	64	32	16	0	25	12	12
2 P 712, Ch, 2 ab	0.001 ml. 4.2 × 10 ⁴ PFU	Vas.	14	4:2	<4	64	16	<2	0	16	2	0
		Sch.	10	4:2	<4	32	16	4	0	12	4	0
		Mor.	7	5:2	<2	16	<2	2	0	12	0	0
		Est.	42	4:7	<2	256	256	128	0	32	32	20
		Pet.	21	4:7	<2	32	256	64	0	2	4	20
		Fol.	14	4:7	<2	2,048	1,024	512	0	320	200	200
		Sti.	10	4:2	<4	1,024	512	128	0	64	32	32
		Dan.	10	2:7	<2	32	16	8	0	16	4	2
		Goo.	61 +	5:7	<2	256	256	64	0	32	32	6
		Tro.	61 +	5:2	<2	32	32	16	0	0	0	2
		Put.	42	5:7	<2	32	128	64	0	0	3	4
		Cro.	42	5:2	<2	128	256	256	0	20	32	32
3 Leon 12 a ₁ b	0.01 ml. 4.3 × 10 ⁵ PFU	Dor.	42	4:7	<2	32	64	64	0	0	0	3
		Nap.	28 +	5:2	<2	2	1,024	64	0	0	32	6
		Eas.	28	5:2	<2	128	128	16	0	4	4	4
		Spo.	21	5:2	<2	<2	64	16	0	0	3	2
		Err.	21	4:2	<2	8	16	8	0	0	2	0
		Br.	14	4:7	<2	16	8		0	4	0	
		Har.	14	3:7	<2	64	64	64	0	4	32	6

It is now two years since the large lots of vaccine, currently used in progressively larger numbers, were first fed to human beings, and tests for persistence of antibody and resistance of the intestinal tract will shortly be carried out. Table IX shows the variation in antibody response of young adults and of antibody levels in individual persons during the first two months after ingestion of only one type of vaccine. The following points are especially worthy of note: (a) differences between low + high-avidity and high-avidity antibody titres at different times after infection; (b) the relatively low titres and occasionally absent high-avidity antibody following infection with the type 3 virus despite many weeks of extensive multiplication in the intestinal tract; (c) the transient antibody response in the three men who received the 10 times smaller dose of the type 1 vaccine, which was also associated with a much more limited extent of viral multiplication than is indicated by the peak titres of virus per gramme of stool that occurred for only a few days.

It is also important to note here that tests on five originally triple-negative children, who received 0.01-ml. doses of the same lots of vaccine seriatim at three-week intervals (1, 3, and 2), showed both more extensive and prolonged viral multiplication and higher titres of high-avidity antibody (Sabin, 1958). Thus, in the case of type 1 all were still excreting virus at 21 days, when further multiplication was cut off by the administration of the type 3 virus, and the high-avidity antibody titres 77 days after feeding were 3,200, 2,000, 2,000, 640, and 640. It is possible that these higher titres may in part at least be the result of the boosting effect of the subsequent infections with the types 3 and 2 viruses. While this may also be the explanation for the high-avidity antibody titres of 16, 16, 50, 64, and 200 against the type 3 virus, the same explanation cannot be invoked for the consistently higher titres of high-avidity antibody (200, 320, 320, 320, and 320) that were found in the five children against the type 2 virus, which was administered last. It is also noteworthy that both the duration of virus excretion and the amount of type 2 virus excreted per gramme of stool were greater in the children than in most of the adults (Sabin, 1958).

Effect of Multiple Doses of Salk Vaccine on Multiplication of Virus in Alimentary Tract and Immunogenic Effect of Orally Administered Attenuated Strains

The question has been raised whether "adequate" immunization with a potent formalinized vaccine of a high proportion of susceptibles in a country might not lead to the elimination of polioviruses from the community (Dick and Dane, 1958), and the relatively low incidence of poliomyelitis in the U.S.A. has been considered by some as reflecting the effect of the Salk vaccine not only on those who received it but also on the total dissemination of the polioviruses in the community. Studies on initially non-immune human beings who had been fed the three types of attenuated poliovirus after two doses of Salk vaccine (Sabin, 1956a, 1957a, 1958; Horstmann *et al.*, 1957a, 1957b) indicated no effect on the multiplication of the virus in the alimentary tract.

In the recent study of Clarke *et al.* (1958) in which type 3 attenuated virus was fed to 14 children who had previously had two doses of formalinized vaccine, 11

had naturally acquired type 3 antibody prior to the injections of the formalinized vaccine, and the other three had no high-avidity antibody; but natural infection with type 3 virus prior to the inoculation of formalinized vaccine cannot be excluded even in them, because no tests for low-avidity antibody were performed. Furthermore, these investigators fed a smaller dose of this virus in hard gelatin capsules, and since there were no controls for this dose and procedure in children without homotypic antibody, it is difficult to evaluate their results. It is noteworthy, however, that their records show that the virus multiplied for 31 days in two of the three children who had no naturally acquired high-avidity antibody and in only 3 of the 11 children with naturally acquired homotypic antibody for periods of 9, 14, and 14 days respectively. In my own tests with a somewhat larger dose of the type 3 virus given by mouth in syrup (instead of capsules), the incidence of infection in young adults with naturally acquired antibody was 50% (Sabin, 1957b).

A study on 25 children without naturally acquired type 1 or type 3 low- or high-avidity antibody (10 type 1 and 15 type 3) who were fed the corresponding attenuated strains after four doses of Salk vaccine was completed in my laboratory early in 1958 (in association with Drs. Krugman, Giles, Barnes, and Wang). The detailed results of this study (quoted in Sabin, 1958) have not yet been published, but have been made available to workers in this field. Four doses of Salk vaccine were used, because five months after the third dose (given about six months after the second) 4 of 9 (44%), originally without type 1 antibody, had no demonstrable type 1 antibody, and 10 of 19 (53%), originally without type 3, had no demonstrable type 3 antibody by the sensitive pH method.

The fourth dose consisted of an especially selected lot of high antigenic potency. Many of the children had titres of 128 and 256 three months after the fourth dose when they were fed 0.01 ml. of the same large lots of type 1 and type 3 attenuated vaccine, used in the studies on children without homotypic antibody. These higher antibody levels had no effect on either the amount or the duration of virus excretion in the stools. Variations in the expected patterns, suggesting interference with multiplication of the poliovirus in the intestinal tract of some of the children, were found to be associated with concurrent infections by various enteric viruses—for example, E.C.H.O. types 7 and 9, adenoviruses types 2 and 16, and two as yet unidentified viruses with properties distinct from the E.C.H.O., Coxsackie, and adenoviruses.

Pharyngeal Poliovirus

Nine of these children (four type 1 and five type 3) received a 10 times larger dose of virus, which on the basis of previous tests in young, non-immune adults was expected to result in virus multiplication in the throat of approximately 80%, and frequent tests for pharyngeal virus were carried out during a period of four weeks. Although all of these children excreted virus in the stools for periods of two weeks or longer, no virus was at any time found in the posterior pharyngeal wall. Since antibody may be expected to be present in the nasopharyngeal secretions of those with titres of about 25 or greater in the blood (Bell, 1948) one might have expected this interference with virus implantation in the throat of those possessing the higher levels of antibody.

However, two of this group of nine children, who had no demonstrable antibody, and one, who had only a titre of 4, at the time of virus feeding also exhibited no virus in the throat.

Despite this failure to correlate the results with the antibody status in the children, the uniformly negative findings in the tests for pharyngeal virus in this group strongly suggest that antibody produced by killed-virus vaccine can interfere with the localization and multiplication of polioviruses in the throat but not in the intestinal tract. It has also been pointed out elsewhere (Sabin, 1956b, 1958) that under natural conditions of infection with "viraemic" strains the high incidence of pharyngeal virus in patients with the clinical disease is more readily explained on the basis of secondary localization from the blood than on primary localization of ingested virus. On this basis one would expect that even minimal amounts of antibody in the blood engendered by killed-virus vaccine would be sufficient to block the secondary localization of "viraemic" polioviruses in the throat.

Those who assume that the throat is the major source of contagion with poliovirus among so-called "sanitary" populations (and the most casual observation indicates that small children of any socio-economic group are highly "unsanitary" human beings) would use such data to support the view that a killed-virus vaccine can influence the extent of poliovirus dissemination in a community. Actually, as has been pointed out elsewhere (Sabin, 1956b, 1958), the pharyngeal poliovirus is swept down into the stomach and is only rarely found in epidemiologically insignificant trace amounts in the mouth and saliva, and therefore cannot under ordinary circumstances be a significant source of contagion to others. Field studies during epidemic (Lipson *et al.*, 1956; Davis *et al.*, 1958) and non-epidemic periods (Fox *et al.*, 1958) have shown that the antibody produced by two or three doses of Salk vaccine does not interfere with the capacity of the individual to disseminate polioviruses.

Viral Multiplication in Intestinal Tract

It has been suggested (Salk, 1957) that an accelerated antibody response to natural infection in those previously immunized with formalinized vaccine may provide long-lasting immunity to paralysis even after demonstrable antibodies had disappeared from the circulation. Previous tests on adults (Sabin, 1957a, 1958) had revealed no evidence of either an accelerated or higher antibody response following infection with orally administered attenuated polioviruses in those who had been previously sensitized either by killed virus vaccine or by natural infection.

A further test of this question was carried out on 24 of the children in our recent study on the effect of four doses of Salk vaccine. Serum specimens were obtained five days after feeding of the attenuated polioviruses (because antibody is first detected in seven days in unsensitized persons), and in not a single instance was the titre of either high- or low-avidity antibody different from that in the serum taken immediately before feeding of the virus. In all instances multiplication of virus in the intestinal tract was later followed by a boost in antibody, but as regards the level of antibody ultimately achieved in these children as compared with those achieved in the initially triple-negative children who were fed the same doses of the

same vaccines seriatim at three-week intervals without prior immunization by Salk vaccine (Sabin, 1958), the results were different for type 1 and type 3. In the type 1 group the very high titres of low-avidity antibody (512 to 4,096) were comparable to those obtained in the triple-negative children, but with few exceptions the titres of high avidity antibody were lower than in the triple-negative children. On the other hand, in the type 3 group the titres of both high-avidity (170–4,096) and low-avidity (256–4,096) antibody were strikingly higher than in the triple-negative children.

These studies clearly show that orally administered attenuated polioviruses are capable not only of greatly boosting the level of antibody but also of imparting the benefits of an intestinal infection in those who had already had four doses of commercially produced formalinized vaccine.

Field Trials Completed and in Progress with Aliquots of Large Lots of Types 1, 2, and 3 Attenuated Vaccines

The large lots of type 1 (L Sc, 2 ab), type 2 (P 712, Ch, 2 ab), and type 3 (Leon 12 a,b) attenuated poliovirus vaccine that are currently being used in field trials on increasingly larger numbers of children were prepared in December, 1956, and tested in small groups by different investigators in 1957 and 1958. The many preliminary tests on the triple-plaque-purified strains used for the preparation of these large lots were previously described (Sabin, 1957a, 1957b). Approximately 22 to 25 litres of each of the three types were prepared at the Merck Sharp and Dohme Research Laboratories in co-operation with Dr. Bettylee Hampil and her associates. Trypsinized rhesus kidney cells grown in 0.5% lactalbumin hydrolysate in Hanks's solution with 2% calf serum were used. After inoculation of the seed virus the maintenance medium consisted of 0.5% lactalbumin hydrolysate in Earle's solution gassed with CO₂ to a pH of about 7.5 without serum.

Ten per cent. of the bottles prepared from each lot of trypsinized monkey kidneys were left uninoculated and observed for the appearance of spontaneous simian viruses. No evidence of spontaneous virus activity was found in any of the original bottles, nor on passage of the harvested fluids to monkey kidney and human amnion cells. The passages in human amnion cells were carried out to check on the possible presence of measles virus. The harvested culture fluids were centrifuged and passed through K 6 and S 1 Seitz filter pads in series at room temperature. The Seitz-filtered fluids constitute the vaccine and have been stored at approximately -20° C.

The tests prescribed by the American National Institutes of Health for aerobic and anaerobic bacteria, for tubercle bacilli in guinea-pigs, for lymphocytic choriomeningitis in mice, and for B virus in rabbits (10 ml. into each of six rabbits for each lot) were all carried out with negative results.

For the identification of the virus in each vaccine, as well as to check for the absence of non-poliomyelitic cytopathogenic viruses, specially potent poliovirus rabbit antisera were used. These rabbit antisera, in 0.1 ml. amounts, were capable of neutralizing approximately 10 million TCD₅₀ of homotypic poliovirus, so that it was possible to test the undiluted vaccine

for the presence of other cytopathogenic viruses. No extraneous cytopathogenic virus was found by this method in any of the large lots of vaccine. The efficacy of this method was emphasized when similar tests on the large lot of Koprowski's type 1 "Chat" vaccine used in the Belgian Congo trials (Courtois *et al.*, 1958) revealed the presence of an unidentified, non-poliomyelitis cytopathogenic virus up to a 10^{-2} dilution of the culture fluid, but not in the higher dilutions.

The intracerebral and spinal tests for residual neurotropism in monkeys were reported previously (Sabin, 1957b; see also Table I). Additional intracerebral tests in 14 monkeys with the 10^{-1} and 10^{-2} dilutions of the type 1 vaccine, in search for evidence of a mixed population or of mutants originating in the monkeys, were also negative. Since these three strains are devoid of neurotropism for the mouse, the absence of this property was checked in each lot of vaccine by inoculating 20 adult mice with 0.02 ml. intraspinally. Although spinal inoculation of chimpanzees is not regarded as a necessary part of the testing of such a vaccine, each of the three lots was tested in three chimpanzees devoid of spontaneously acquired antibody for all three types of poliovirus. Chimpanzees with spontaneously acquired antibody for any of the three types of poliovirus might also have some lesions in the spinal cord, and therefore are not suitable for this type of test. Each chimpanzee was inoculated in the region of the lumbar enlargement (located by drawing a line across the lowest tips of the scapulae with the arms completely adducted) with 0.2 ml. of the undiluted culture fluid through an 18-gauge needle. Neither paralysis nor lesions in the spinal cord were found in any of the nine chimpanzees observed for a period of at least three weeks, and multiple sections through the lumbar cord indicated that the inoculum had been properly placed in the grey matter.

Potency of the Vaccines

The potency of each vaccine is determined by titration in cynomolgus monkey kidney tissue culture tubes, using 10 tubes for each tenfold dilution or five tubes for each threefold dilution. Comparative tests showed that rhesus cells yield lower titres (0.7 log for type 1, 0.3 log for type 2, and no significant difference for type 3), and that titres consistently higher by 0.3–0.5 log are obtained when the serum contained in the growth medium is leached out of the cells by leaving them in contact with serum-free medium for at least two hours. This is then replaced with fresh serum-free medium prior to the addition of the virus. The medium consists of 5% lactalbumin hydrolysate previously neutralized with NaOH to a pH of about 7.5 and diluted to 0.5% in Earle's solution (0.22% NaHCO_3), the final mixture being gassed with CO_2 to a pH of about 7.5. The virus-inoculated tubes are kept in a roller drum and the final readings are made eight days after inoculation. The Hsiung–Melnick technique in stoppered bottles was used for plaque counts, but the following modification in adsorption of the virus yielded two to three times higher counts with these particular attenuated strains: (a) the acid medium must be washed away by 5 ml. of alkaline medium, which is left on the cell sheet for about an hour and then removed prior to the addition of virus for adsorption; and (b) the virus must be diluted in maintenance medium containing 0.22% NaHCO_3 and

at least 0.3 ml. added to each bottle in order to prevent the development of a highly acid fluid during the period of adsorption.

The type 1 vaccine has maintained its potency despite bottling at room temperature and storage at about -20°C . for two years—the most recent titres on various ampoules having yielded values of $10^{7.8}$ to $10^{8.1}$ TCD_{50} per ml. for the type 1 vaccine. The type 2 and type 3 vaccines have dropped somewhat in potency during this period from $10^{7.7}$ to $10^{7.3}$ TCD_{50} per ml. for type 2, and from $10^{7.9}$ to $10^{7.4}$ TCD_{50} per ml. for type 3. Tests carried out by Dr. P. A. D. Winter in my laboratory indicated that the 1:10 dilution of the vaccine used for feeding can be maintained in wet ice for at least a week without loss of titre.

The original tests that I carried out in 110 young adults and children were reported previously (Sabin, 1957b, 1958). Since that time I also fed these lots to 33 children and adults in various families, and to the 26 children in the study on the effect of multiple doses of Salk vaccine. The most detailed small-scale study on 178 children and adults in Leyden, Holland, was carried out in 1957 and 1958 by Professor J. D. Verlinde and his associates, who have recently published their results (Verlinde *et al.*, 1958).

Dr. M. Ramos-Alvarez and Professor Federico Gomez, of the Hospital Infantil in Mexico City, have carried out stepwise studies on progressively larger numbers of children, most of them under 5 years of age since May, 1957, and reported their results at the International Congress on Tropical Medicine at Lisbon, Portugal, in September, 1958. They began with 70 children, then increased the number to 200, and when no untoward reactions were observed in them or their contacts at home over a period of several months, they began early in 1958 a small-scale field trial on an additional 3,500 children who were given the three types of vaccine *seriatim* as described earlier. Although the number without homotypic antibodies for any one type varied with the age of the children, approximately 40 to 50% of the entire group were without homotypic antibodies prior to the ingestion of the vaccine. A most careful clinical surveillance failed to reveal any untoward reactions or illnesses attributable to the vaccines in these children or their contacts, and these investigators, in co-operation with the Ministry of Health in Mexico, have decided to start a much larger trial of aliquots of the same vaccines on approximately 500,000 children in three Mexican cities in February, 1959.

In June, 1958, Dr. Guillermo Contreras and Professor Arturo Scroggie, of Santiago, Chile, carried out a small-scale trial in 50 newborn children who received the three types of vaccine *seriatim* (1, 3, and 2 at four-week intervals). The results of antibody tests are not yet available, but they found no untoward effects in the children or their contacts (personal communication).

In October, 1958, Professor James H. Hale, of the University of Malaya, in Singapore, in co-operation with Dr. Doraisingham and others, of the Ministry of Health in Singapore, initiated a large-scale field trial in 200,000 children 3 months to 10 years of age. A serological survey previously carried out by Professor Hale indicated that the age at which 50% of the children had acquired antibodies for any one type was 7 to 10 years. The type 2 vaccine was administered first

because Singapore was at the time in the midst of a growing epidemic of exclusively type 1 paralytic poliomyelitis. The results soon to be reported by Professor Hale and his associates should be of special interest because of the light they may throw on the extent of interference and protection that an infection with type 2 virus may exert against the paralytic effects of an epidemic strain of type 1 poliovirus, not only in the vaccinated children but also in the remainder of the population. Both Professor Hale and I were against using the type 1 attenuated poliovirus in the midst of a type 1 epidemic, before the safety of the vaccine had been established on a sufficiently large scale under non-epidemic field conditions.

Another large-scale field trial with aliquots of these large lots of vaccine designed to test the effectiveness of combined killed-virus and live-virus vaccine immunization was begun by the Ministry of Health in Czechoslovakia in December, 1958, under the direction of Drs. Skovranek, Raska, Prochazka, Zacek, and Slonim. Almost all children under 12 years of age in Czechoslovakia had received Salk vaccine (predominantly of Canadian manufacture) in 1957 and 1958. The approximately 143,000 children 2 to 7 years of age in four different regions of the country involved in this trial had previously received three 0.2-ml. intracutaneous doses of formalinized vaccine at the proper intervals in 1957 and 1958. The type 1 oral attenuated vaccine was administered during the course of a few days beginning December 15, 1958, and the type 3 vaccine beginning January 12, 1959; the type 2 vaccine is scheduled for four weeks after the type 3. Serological surveys prior to and after the administration of these vaccines, as well as the careful long-range clinical and virological surveillance of the vaccinees and their contacts, that have been organized by the Czechoslovak investigators should provide us with important information of the greatest significance for the ultimate control of poliomyelitis.

Approximately 225,000 doses of aliquots of each of the three lots of vaccine have been supplied to Professors Chumakov and Smorodintsev in the U.S.S.R. for large-scale trials that began late in 1958. Small-scale studies by Professor J. Fox and his associates, of Tulane University, and by Professor J. R. Paul and his associates, of Yale University, in the U.S.A., and by Professor M. Kitaoka, of the National Institute of Health in Japan, with aliquots of the same lots of vaccine are also expected to contribute valuable information for the ultimate evaluation of the behaviour of these particular strains in human beings.

The expert committee on poliomyelitis of the World Health Organization (1958), in the portion of its July, 1957, report dealing with live virus vaccine, stated: "It is the carefully considered opinion of the committee, therefore, that the information which is now badly needed can only be obtained from field trials carried out on a larger scale than has been possible in the past," and laid down specifications for the strains of virus and the lots of vaccine to be used in such trials as well as the manner in which they should be performed. The large lots of vaccine here described fulfil these specifications, and it is hoped that the large-scale field trials now in progress, and still others yet to come, may provide the answer regarding the ultimate safety and usefulness of live virus vaccine in the prevention and eradication of poliomyelitis.

Summary

The reasons why one cannot speak of a return to virulence in polioviruses, based merely on the finding that large doses of poliovirus produce paralysis in a varying proportion in intracerebrally inoculated monkeys, are analysed and discussed. The proper measurement of different grades of neurotropism in monkeys, only one of the factors determining the virulence of ingested polioviruses in higher primates, is dependent on the employment of certain specified techniques which are suitable for measuring the varying capacity of polioviruses for spreading from one group of initially exposed susceptible cerebral or spinal neurones to another. Although the best attenuated strains, developed thus far, still have a limited residual neurotropism for the spinal motor neurones of the monkey, they produce neither paralysis nor lesions in chimpanzees properly inoculated in the spinal cord. Furthermore, viruses that are distinctly paralytogenic after intracerebral inoculation of moderate doses in monkeys are not paralytogenic on proper spinal inoculation of large doses in chimpanzees.

Additional data on the neurotropism of the polioviruses excreted in the stools following ingestion of the most highly attenuated currently available strains indicate: (a) that a certain proportion of the viral population (more so with type 3 than types 1 and 2) is less attenuated than that which was ingested, that portion being selectively detected by inoculation in the nervous system of monkeys; (b) that the less attenuated virus is not selectively propagated in the human alimentary tract, as is evident from the disappearance of such virus after prolonged multiplication in the intestinal tract of the original vaccinees, and the failure to find progressive increase in neurotropism after further multiplication in the intestinal tract of naturally exposed contacts, or after repeated experimental passages in human beings. These findings are the main reason for utilizing the most highly attenuated strains, possessing an adequate capacity for multiplication in the human intestinal tract, for vaccinees to be used in large-scale trials in human beings.

The antibody response to the feeding of live attenuated polioviruses has been shown to depend on the dose, strain, and extent of multiplication in the intestinal tract. Persistence of detectable antibody is not dependent on the titre achieved, and resistance of the alimentary tract has been shown to be present even in the absence of readily demonstrable antibody.

Multiple doses of Salk vaccine, currently produced commercially in the U.S.A., often fail to produce an adequate antibody response and the persistence of the antibody is dependent on the level originally produced. However, even high levels of antibody produced in some children by four doses of Salk vaccine failed to influence in any way the extent of viral multiplication in the intestinal tract, although localization and multiplication in the throat was apparently prevented. The available evidence, however, indicates that virus in the throat is of little or no importance in the transmission of infection to others. No evidence was found that previous immunization with Salk vaccine results in an accelerated antibody response to multiplication of orally administered polioviruses, and there is therefore no theoretical basis for expecting long-lasting immunity from a formalinized vaccine after the initially produced antibody has disappeared.

Studies on progressively increasing numbers of children, with aliquots of large lots of vaccine prepared

in December, 1956, with the most highly attenuated currently available strains of each of the three types, have thus far produced no untoward reactions in vaccinees or their contacts. Spread of the vaccine viruses to others is an accepted fact in the immunization with live poliovirus vaccines, and this problem is not being "side-stepped" but is rather being carefully studied in various trials. The trials on some hundreds of thousands of children—some already completed, others now in progress, and still others to be initiated in 1959—carried out in accord with the stipulations of the Expert Committee on Poliomyelitis of the World Health Organization should provide us in the near future with the information necessary for a decision regarding the place of orally administered live poliovirus vaccines in the prevention and eradication of poliomyelitis.

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RESPONSE OF INFANTS TO A THIRD DOSE OF POLIOMYELITIS VACCINE GIVEN 10 TO 12 MONTHS AFTER PRIMARY IMMUNIZATION

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In our first study (Perkins, Yetts, and Gaisford, 1958) we reported that when infants aged 10 weeks or less were given a primary course of two doses of poliomyelitis vaccine the antibody response was in most cases unsatisfactory. This was due principally to the inhibiting effect of maternally transmitted antibodies, but also to the fact that a stronger antigenic stimulus is necessary in the newborn period to produce results comparable with those obtained in later infancy.

Despite the unsatisfactory results it was thought possible that, in at least some of the infants, primary immunization might have occurred but that we had been unable to demonstrate this because the increase in antibody level had been masked by the maternally transmitted antibodies. It was therefore decided to recall the infants approximately a year later and, from a study of the response to a reinforcing dose, obtain further information on the extent of any basal immunity which had been induced.

Procedures

Immunization.—Of the 88 infants previously studied, 80 were available. They were given 1 ml. of vaccine intramuscularly 10 to 12 months after their second dose. The vaccine used was Glaxo batch 13, which had been shown to have satisfactory antigenic activity by the routine test in monkeys (Biological Standards Control Laboratory, 1957). The infants, who were 1, 6, and 10 weeks old respectively at the time of injection of the first dose of vaccine, were designated as groups A, B, and C. In the present investigation there were 29 in group A, 28 in group B, and 23 in group C.

Titration of Sera.—The sera were titrated for poliomyelitis-neutralizing antibodies to each of the three virus types by the method previously described (Biological Standards Control Laboratory, 1957), which has been shown to give reproducible results (Perkins, Sousa, and Tobin, 1958). Fourfold serial dilutions of sera were used, the test dose of virus was approximately 100 TCID₅₀, and each serum-dilution/virus mixture was inoculated into two monkey-kidney-cell culture tubes. The two samples of serum from each infant were titrated in parallel in the same test, together with a repeat titration of the serum sample taken 14 to 21 days after the second dose. In the case of one infant in group B it was not possible to determine antibody levels to types 2 and 3 virus. All titres are given as the dilution of serum in the serum-virus mixtures before addition to cell cultures.