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IPV-Vero Vaccine Induces a Strong Booster Reaction and is Well Tolerated in Adults

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A phase 1–2 trial was conducted in 48 adults to study safety and immunogenicity of an inactivated poliovirus vaccine produced using Vero cells (IPV-Vero). Participants received 2 intramuscular injections with IPV-Vero (40-8-32 D-Ag units) 4 weeks apart. IPV-Vero was well tolerated, and induced strong antibody responses in all participants. At least an 8-fold titre rise against all 3 types of poliovirus was found within 1 week of the first vaccination, indicating a strong secondary response in primed individuals. Two days after the first vaccination, there was no indication for such a booster reaction. The second vaccination 4 weeks after the first dose did not further increase antibody levels, indicating that an immune plateau had been achieved after the first vaccination. The second vaccination was not reactogenic despite the presence of these high pre-vaccination antibody levels. We conclude that IPV-Vero is well tolerated and strongly immunogenic in adults. In pre-immune adults 1 dose is enough to induce an impressive booster reaction.

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INTRODUCTION

Inactivated poliovirus vaccine (IPV) has an excellent and long-standing record of safety and efficacy. Its persistent immunogenicity correlates with excellent individual protection against poliomyelitis, as shown in several European countries, including The Netherlands and Sweden. Endemic poliomyelitis disappeared after introduction of trivalent IPV in national immunization programmes and more than 95% of the vaccinated population have high and persistent levels of poliovirus (PV) neutralizing antibodies (1–3). In The Netherlands, the most recent epidemics (in 1978 and 1992–93) occurred in small communities of people who had rejected vaccinations for religious reasons and are attributed to their choice not to vaccinate rather than to vaccine failure (4).

For the Netherlands vaccination programme, our institute (RIVM) supplies quadruple DPT-IPV and triple DT-IPV vaccine. Currently, the IPV component is produced using monkey kidney (MK) cells for virus culture (5). RIVM explored another cell substrate in which PV can be propagated effectively on a large scale. Vero cells, also derived from monkey kidneys, have advantages over MK cells. They are reliable and cheap, better standardized, have a smaller risk of being contaminated than monkeys and their kidney cells and further use of primates for virus propagation is unnecessary. Vero cells are widely used by others for production of live and killed viral vaccines (OPV, IPV and rabies) (6). IPV produced on Vero cells (IPV-Vero) is considered safe and potent, as reviewed by Vidor et al. (7).

The present study in adults was the first in a series to investigate the safety and antibody response to IPV-Vero. Further studies in infants to compare the immunogenicity of IPV-Vero and IPV-MK are ongoing.

MATERIALS AND METHODS

Vaccine

IPV-Vero (lot E9433B) was produced using standard methods (5), and contained per dose of 0.5 ml: formalin-inactivated poliovirus (strains Mahoney, MEF-1 and Saukett), type 1, 2 and 3: 40-8-32 D-antigen units respectively, and formaldehyde: 0.025 mg in phosphate buffer. The vaccine was injected in the deltoid or triceps muscle.

Study

This combined phase 1–2 study was descriptive and without control vaccine. The study has been approved by the Medical Ethical Review Board of the University Hospital Utrecht, and was performed between November 1994 and February 1995, at the Department of Intensive Care and Clinical Toxicology of the University Hospital Utrecht, The Netherlands.

Volunteers were recruited by advertisements in the "Utrecht University Weekly" magazine on University bulletin boards and by mailing volunteers from former clinical trials. Thus, most volunteers were students. After screening for inclusion and exclusion criteria, participants gave written informed consent.

Vaccinations were given at day 0 and 28. Venous blood samples for antibody determinations were taken at day 0, 2, 7, 28 and 56 from the first vaccination. Reactogenicity was assessed at days 0, 2, 7, 28, 30 and 56.

Antibody response

Serum neutralizing (SN) antibody levels were determined with Vero cells as indicator cells, and wild PV strains for challenge (8), the same strains as used for vaccine production. After inactivation, sera were investigated in a 2-fold dilution series up to 17 dilution steps. SN activity is expressed as 2 log reciprocal titres (e.g. 5 means $1/32 = 2^{-5}$). For statistical comparison of antibody levels the Kruskall-Wallis test was used, with EpiInfo (9).

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Adverse events

After vaccination, volunteers were observed for 1 h for heart rate, blood pressure and temperature. Temperatures were measured orally at days 0, 2, 28, and 30. Local and systemic symptoms were studied by observation and questionnaires. The injection site was examined for inflammatory symptoms.

Participants kept a diary about their well-being and possible adverse experiences, for discussion at subsequent visits, at which the participant was asked for general well-being, inability to perform normal daily activities, headache, gastrointestinal complaints, joint complaints and skin abnormalities.

Reactions were graded according to severity. A mild reaction was perceived by the participant but was easily tolerated. A moderate reaction was giving enough discomfort to interfere with normal daily activities.

RESULTS

Study population

A total of 48 volunteers were enrolled and 47 completed the study (18 males and 30 females; mean age 23.5 y, range 18–39 y). One participant was excluded during the study due to protocol violation, from another the 2 last blood samples were excluded due to an insufficient interval between vaccinations.

Antibody response

The results of immunogenicity are summarised in Table I. Cumulative distributions of SN titres are shown in Fig. 1. Not unexpectedly, all participants had detectable antibodies before vaccination. A titre of ≥ 3 (1/8 or higher) is considered to be protective. To PV type 3 (PV3) an SN titre below 3 was found in only 3 individuals and to PV1 and PV2 all had initial titres ≥ 3.

Two days after the first vaccination there was little difference from pre-vaccination titres. However, after 7

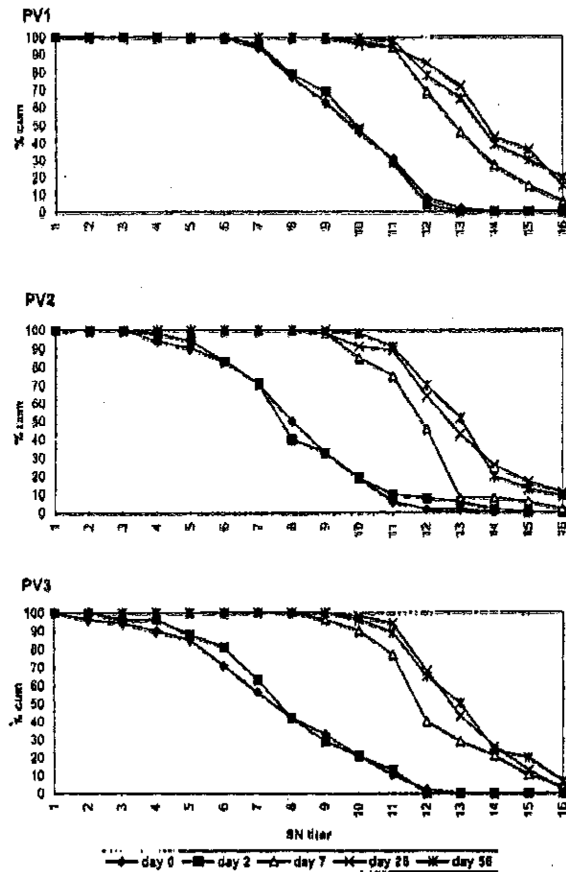

Fig. 1. Poliovirus neutralizing antibody response to IPV-Vero vaccine. The percentage reverse cumulative frequency of serum neutralizing titres is given per poliovirus serotype. Vaccinations were given on days 0 and 28 and blood samples were taken on days 0, 2, 7, 28 and 56.

Table I. *Summary of serum neutralizing antibody response to IPV-Vero in adults, vaccinated on day 0 and day 28*

		Day 0		Day 2		Day 7		Day 28		Day 56
PV1										
	% ≥1/8	100		100		100		100		100
	gmt	9.3	ns	9.3	*	12.5	ns	13.4	ns	13.3
	SD	1.8		1.7		1.7		1.9		1.8
PV2										
	% ≥1/8	100		100		100		100		100
	gmt	7.5	ns	7.6	*	11.6	ns	12.4	ns	12.5
	SD	2.2		2.4		1.8		2.0		1.7
PV3										
	% ≥1/8	93.8		95.8		100		100		100
	gmt	7.0	ns	7.3	*	11.7	ns	12.4	ns	12.5
	SD	2.7		2.3		2.0		1.6		1.8
	$n =$	48		48		48		47		46

gmt, geometrical mean titre and SD, standard deviation are expressed as a 2 log reciprocal titre (e.g. 5 means $1/32 = 2^{-5}$). Titre differences for successive examination days (day 2 vs day 0, etc.) were evaluated for statistical significance using the Kruskall-Wallis test.
* $p < 0.000001$.
ns, $p > 0.01$.

Table II. *Adverse events after IPV-Vero at day 0 and day 28 in adults (n = 47)*

(a) General symptoms Day of examination	#1 0	2	7	#2 28	30	56
General well-being	0	0	0	0	0	0
Normal daily activities	0	1	0	0	0	0
Headache	0	1	0	1	1	0
Gastrointestinal complaints	1[a]	1[a]	1[a]	1[a]	2[a]	0
Joint complaints	0	0	0	0	0	0
Skin abnormalities	0	1	0	0	1	0
Any general symptom	1	4	1	1	4	0
(b) Local symptoms Day of examination	**#1 0**	**2**	**7**	**#2 28**	**30**	**56**
Redness	5	0	0	10	0	0
Swelling	1	0	0	0	0	0
Itching	0	0	0	0	1	0
Warmth	0	0	0	0	0	0
Pain	0	2	0	0	5	1
Muscle stiffness	1	35	0	1	14	0
Any local symptom[b]	6	36	0	11	18	1

All local symptoms were of mild intensity. All general symptoms were mild except in one participant (underlined) who had a moderate headache.

[a] All occurring in one volunteer, and regarded as not causally related to vaccination.

[b] Some participants had more than one symptom.

days all participants showed at least an 8-fold (3 titre steps) increase for PV1 antibodies, and a more than 16-fold (> 4 titre steps) increase for PV 2 and 3. This was also found in the 3 individuals with 'non-protective' pre-titres: they showed in 7 days strong booster reactions, from 1–10, 2–13 and 1–14, respectively. Apparently, these individuals had excellent immunological memory.

The second vaccination added little to the SN titres which had been reinforced by the first vaccination in these pre-immune participants. Maximal immunity has already been achieved within 1 week of the first vaccination.

Adverse events

The results regarding safety are summarised in Table II. Severe and acute reactions did not occur. The highest recorded temperature was 37.6°C, recorded twice out of 564 measurements. General symptoms were reported by 5 subjects (11%) and local reactions (mainly muscle stiffness) by 42 (89%). The reactions encountered were mild in intensity. Only 1 participant complained of headache with moderate intensity.

Notably, reactions were also very mild after the second vaccination, if they occurred at all, even while all participants had high antibody titres at that time. Arthus reactions did not occur.

DISCUSSION

In this evaluation of the effects of IPV-Vero vaccination very strong antibody responses were observed in all participants. At least 8-fold titre rises against all 3 types of poliovirus occurred within 1 week of the first vaccination, indicating a strong secondary response in already primed individuals. Two days after the first vaccination there was not yet any indication of the booster reaction. The second vaccination after 4 weeks did not further increase the level of antibodies, indicating that an immune plateau had been reached already after the first dose. The strong antibody reaction is not surprising because participants were from the generation of the Dutch population in which more than 90% have received at least 3 childhood vaccinations against poliomyelitis, which is reflected by a high antibody prevalence in this age group (2) and high prevaccination antibody levels among the participants. Therefore, adults are not suitable to investigate a primary antibody response; studies in infants are ongoing.

The vaccine under study is very similar to the IPV-MK currently used in the Netherlands. The only difference is the cell substrate for virus propagation. Biochemical and immunochemical parameters, and *in vivo* potency in rats indicate immunogenic properties equivalent to IPV-MK. It has been clearly demonstrated that immunogenicity in humans correlates with the concentration of D-antigen in the vaccine (10, 11). The optimal dose of trivalent IPV contains 40-8-32 D-Ag units of the 3 types of poliovirus, respectively, which has been determined with IPV-MK in extensive investigations in several countries, both as IPV and as combined DPT-IPV (10–16). The serological response to IPV-Vero was expected to be similar. In this study, the current 40-8-32 D-Ag content per dose appeared very strong indeed. Although a formal comparison with immunogenicity

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of RIVM IPV-MK was not done, we regard the 40-8-32 D-Ag content, similar to Vero-produced IPV from another manufacturer (7), appropriate for use in adults. Studies comparing IPV-Vero and IPV-MK are ongoing.

The 2-dose schedule used here is mainly chosen to study safety aspects. The presence of high pre-vaccination antibody titres did not augment the prevalence of adverse reactions after the second dose. Arthus reactions may occur in hyper-immune persons after vaccination with diphtheria and or tetanus toxoids, but may be also associated with impurities of such vaccines given repeatedly (17). Such reactions are caused by immune-complex formation between the vaccine antigen and high levels of the corresponding antibodies, but we did not encounter them. Compared with toxoids, the amount of protein in IPV is much lower, and its purity better. The vaccine is therefore useful for the rapid induction of immunity, with a low occurrence of side-effects and without the possibility of spreading vaccine virus (as oral polio vaccine does). This makes IPV suitable for use during epidemics, e.g. in hospitals, both for personnel and polio-non-immune patients.

We conclude that IPV-Vero is a well-tolerated and potent immunogenic vaccine in adults. In pre-immune adults 1 dose is enough to induce an impressive booster reaction.

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A CONCEPT OF THE MECHANISM OF IMMUNITY FOR PREVENTING PARALYSIS IN POLIOMYELITIS*

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The principles of immunization, as they appear to apply to the problem of poliomyelitis, have been discussed previously. It is the purpose of this communication to elaborate upon a concept of the dynamics of immunity to paralysis as it has been deduced from certain epidemiologic observations and from studies with noninfectious vaccines. By way of summary, the essential features of the immunologic reaction, in man, following an injection of formaldehyde-treated poliomyelitis virus vaccine, are shown in FIGURES 1, 2, and 3. FIGURE 1 illustrates the level of the antibody response elicited by three doses of vaccine given in a five-week period with the second dose having been administered two weeks after the first. A distinction between the primary vaccination effect, and the secondary, or booster, effect is seen in the comparison of the left-hand and the right-hand frames in FIGURE 1. Persons who have demonstrable antibody from a previous natural infection respond with higher levels of antibody than do those who give no evidence of having had a prior immunologic experience with the respective virus types.

In studies with poliomyelitis vaccines, the same phenomenon has been observed as with other vaccines, where a two-stage procedure is required to raise the concentration of serum-antibody to the very high levels that can be achieved. It has been observed also that a relatively long interval is required between primary and secondary stimulation before the full booster effect can be elicited in man. The minimal interval in man appears to be a number of months. In monkeys, the effect seems to be fully developed in about four weeks. In the mouse, the optimal interval can be measured in days. The relative ineffectiveness of multiple inoculations, when the several doses are given at intervals too short to produce a maximal effect, is illustrated in FIGURE 2. If, however, primary immunization had been adequate, and if a sufficient interval had elapsed between primary and secondary stimulation, then merely a single dose appears to be sufficient to produce a maximal response, as is shown in FIGURE 3.

Present evidence indicates that antibody induced with a noninfectious antigen is not evanescent and, also, that the effects of primary immunization are such that subsequent revaccination, at intervals of up to at least two years, results in sharp rises of antibody to heights comparable to, or beyond, those observed in persons who have had a natural infection. Thus it would appear that man reacts to the poliomyelitis antigen in accordance with the same laws that appear to govern the reaction to other antigens.

The Significance of the Immunologic Complexity of the Poliomyelitis Viruses

In the past several years, many investigators have reported evidences suggesting the existence, in viruses of one type, of antigenic components that

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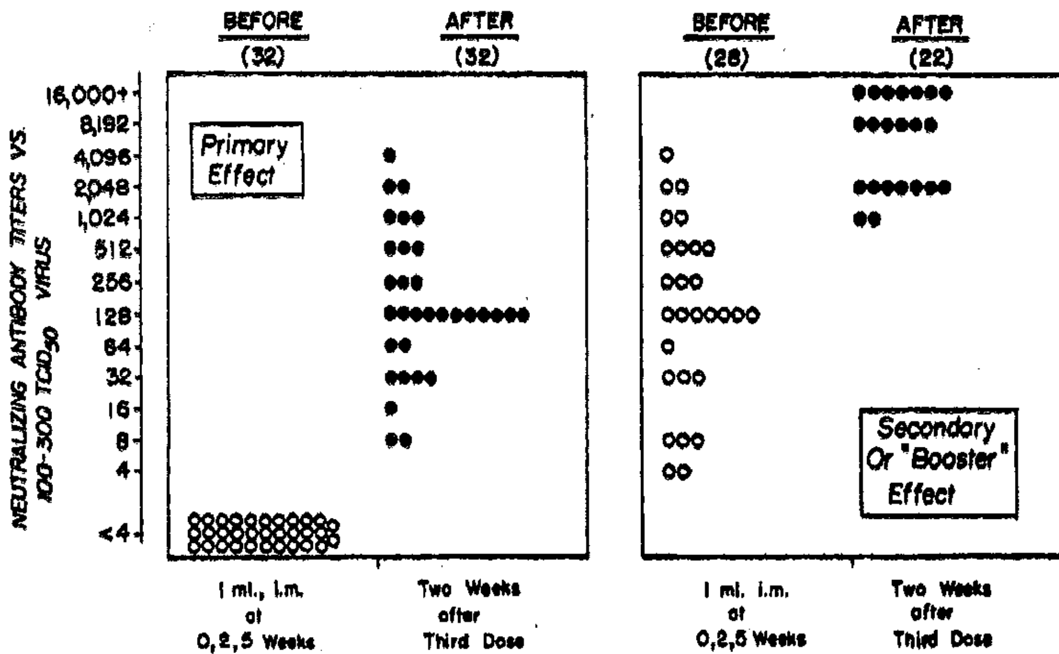

FIGURE 1. Distribution of types 1, 2, and 3 antibody titers before and after vaccination with aqueous trivalent poliomyelitis vaccines. Persons with no demonstrable antibody before vaccination (left); and persons with some antibody from previous nonparalytic infection (right).

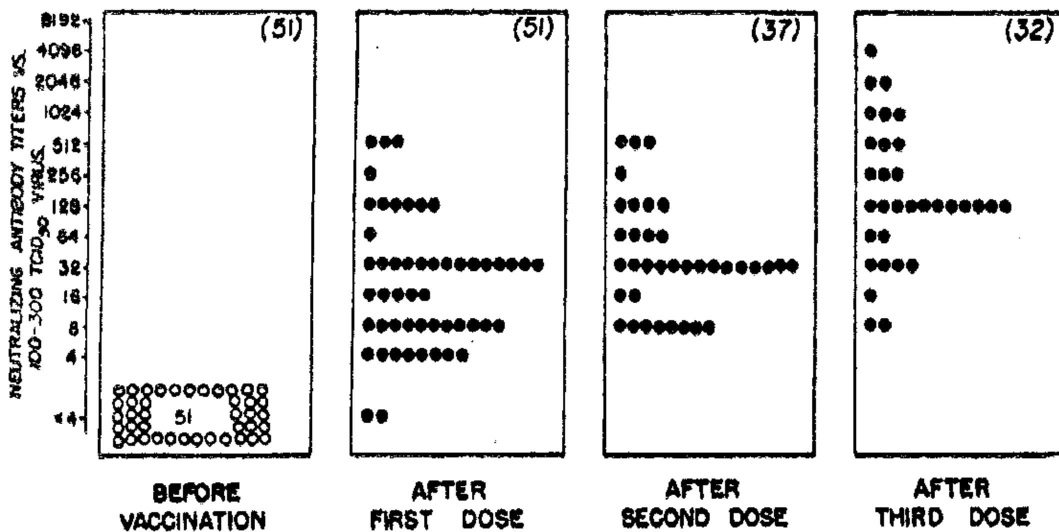

FIGURE 2. Primary vaccination effect. Influence upon antibody level of each of three doses spaced at intervals of two and five weeks after the first dose.

characterize the predominant antigen of one or both of the other two types. Further evidence for this has been obtained in the course of our studies in man. These observations have furnished clues to the probable mechanism whereby, under natural circumstances, paralytic poliomyelitis is controlled by immunologic means. I should like to summarize the essential features of the observations that have been made as they bear on this question.

First, I should like to show the extent to which the immunologic response is different in persons who have no antibody to any of the three types prior to vaccination, as compared with individuals who possess antibody for one or

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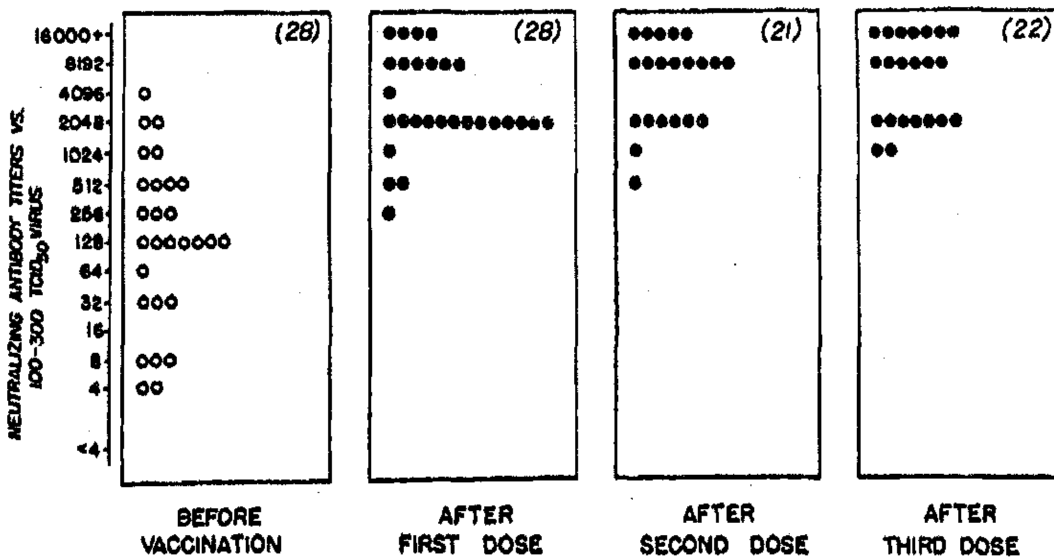

FIGURE 3. Secondary or "booster" effects in persons who have had a natural infection. Influence upon antibody level of each of three doses spaced at intervals of two and five weeks after the first dose.

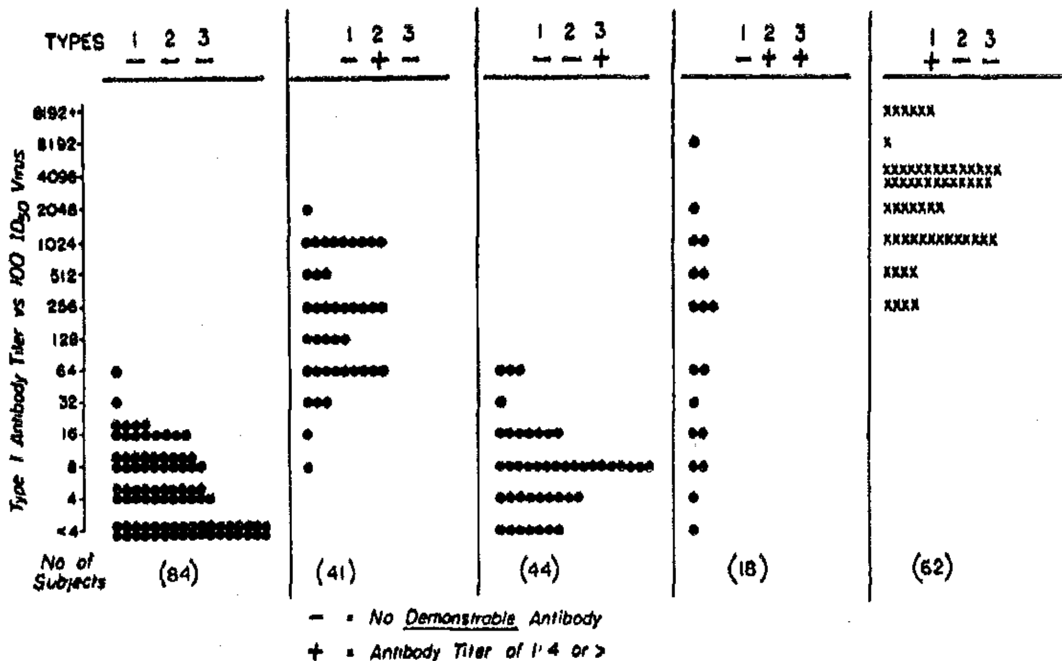

FIGURE 4. Type 1 antibody response to first dose of vaccine in persons with different antibody patterns of prevaccination.

more of the three known types. A comparison of the different degrees of response to the type 1 component of the vaccine, as observed in groups of such persons, all of whom, prior to vaccination, had no demonstrable type 1 antibody, is shown in FIGURE 4. It is to be emphasized that the antibody responses here recorded are those observed after the *first* dose of vaccine. It would appear from these data that persons who had a previous type 2 infection (as indicated by the presence in their blood serum, at the time of vaccination, of antibody for the type 2 virus) responded with much higher levels than did persons who had no antibody to any of the three types. Persons who had a

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prior type 3 infection did not react to the same degree as did those who had a prior type 2 infection. The group with type 3 antibody before vaccination reacted not too differently from individuals who had no previous immunologic experience whatever. Those who, prior to vaccination, had antibody for both types 2 and 3 virus seemed to react as if they had either a prior type 2 *or* type 3 infection. Persons who had a measurable amount of type 1 antibody before vaccination responded with a full booster effect.

It would seem from the data in FIGURE 4 that the naturally occurring type 2 viruses that were the cause of the infection in these individuals must have possessed a sufficient amount of type 1 antigen to have produced the degree of immunologic hyperreactivity that was revealed by the effect of vaccination. Infection with the type 3 viruses, on the other hand, seemed to have produced a barely perceptible sensitizing effect.

When a comparison is made of the response to the first dose of vaccine in persons who do, or do not, possess type 1 antibody before vaccination, one can recognize four grades of reactivity, depending upon the nature of the previous immunologic experience. These observations suggest that the different influences probably depend upon quantitative factors—*i.e.*, the amount of type 1 antigen involved in the previous immunologic experience.

Similar data on the type 2 antibody response, in persons with different previous immunologic experiences, are summarized in FIGURE 5. From the degree of antibody response to the first dose of vaccine in persons with no prevaccination antibody to any of the three types, as compared with the response among those who had a prior type 1 infection, it is clear that the earlier type 1 experience had induced a significant degree of heightened reactivity to the type 2 component of the vaccine. This suggests that the naturally occurring type 1

— No Demonstrable Antibody
+ Antibody Titer of 1:4 or >

FIGURE 5. Type 2 antibody response to first dose of vaccine in persons with different antibody patterns of prevaccination.

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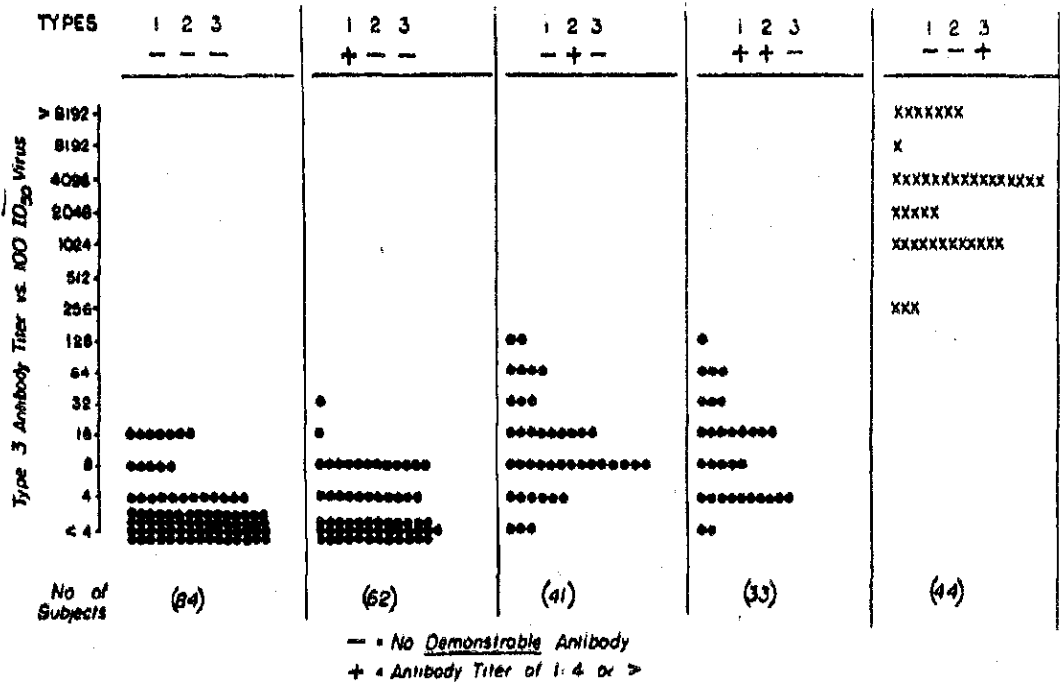

FIGURE 6. Type 3 antibody response to first dose of vaccine in persons with different antibody patterns of prevaccination.

viruses must have possessed some of the antigenic configurations that distinguish the type 2 virus. A comparison with the data in FIGURE 4 suggests that a prior type 2 infection may have exerted a somewhat more marked effect on type 1 responses as compared with the effect of type 1 infection upon type 2 vaccine response. In contrast to the absence of any influence of a previous type 3 infection upon the subsequent type 1 response (FIGURE 4), there is observed in FIGURE 5 a significant influence of the prior type 3 infection on type 2 responsiveness. In terms of degree, it would appear that the influence of the prior type 3 infection was only slightly less than was the effect of the prior type 1 infection. The presence of antibody for both type 1 and type 3 viruses seems to have caused a heightened reactivity to type 2 equal to, or greater than, the effect of either a type 2 or type 3 infection alone.

FIGURE 6 illustrates the type 3 response and, since the type 3 component of the vaccine employed in this particular study was less potent than the types 1 and 2 components, the height of the primary response reflects this difference. The amount of antigen present, however, was still sufficient to induce the sharp booster effect in persons who possessed type 3 antibody from a natural infection. The absence of any appreciable effect, of a previous type 1 infection, on type 3 reactivity is clearly evident. A previous type 2 infection, however, did measurably influence type 3 responsiveness.

The relationships observed in FIGURES 4, 5, and 6 are summarized in FIGURES 7 and 8. They indicate that there is little sharing of specific antigenic configurations in the constituents of types 1 and 3 virus, but there must be a substantial amount of type 1 antigen in the type 2 virus, and a definite, but lesser amount, of the type 2 antigen in the type 1 virus. The quantity of type 2 antigen in the type 3 viruses, and of type 3 antigen in the type 2 viruses, is

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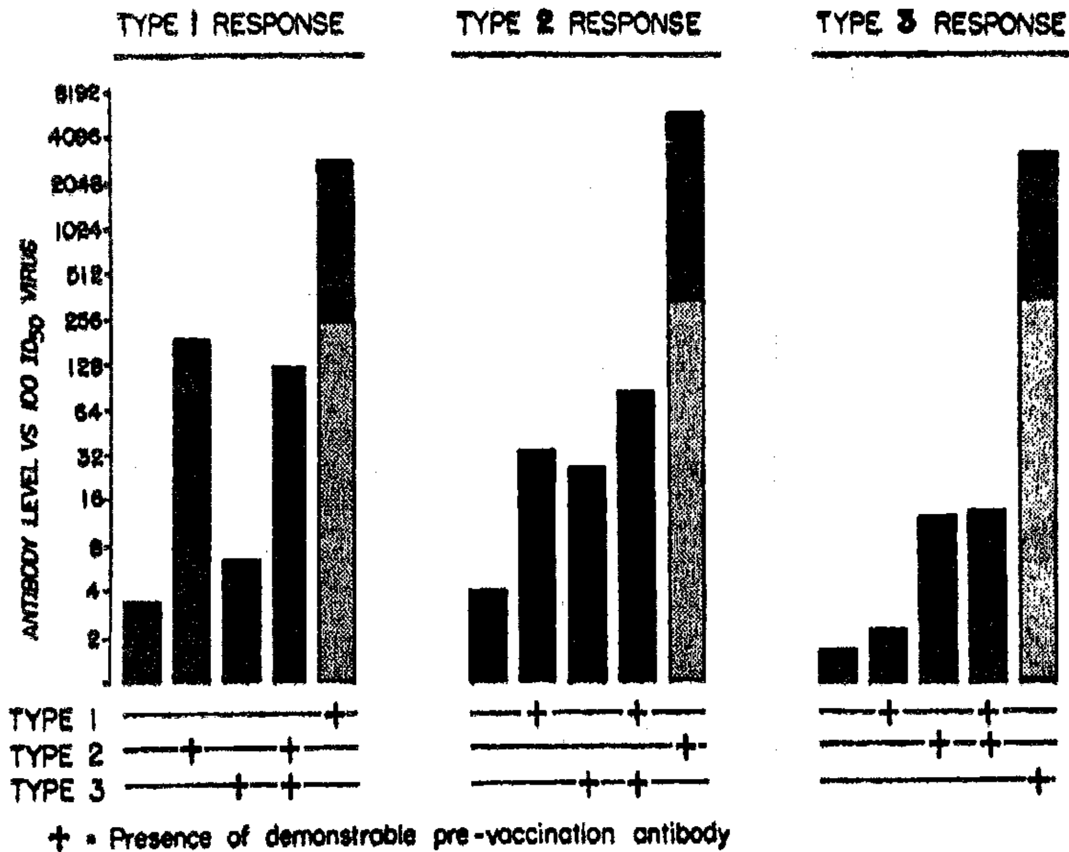


FIGURE 7. Geometric mean of antibody response to first dose of vaccine.

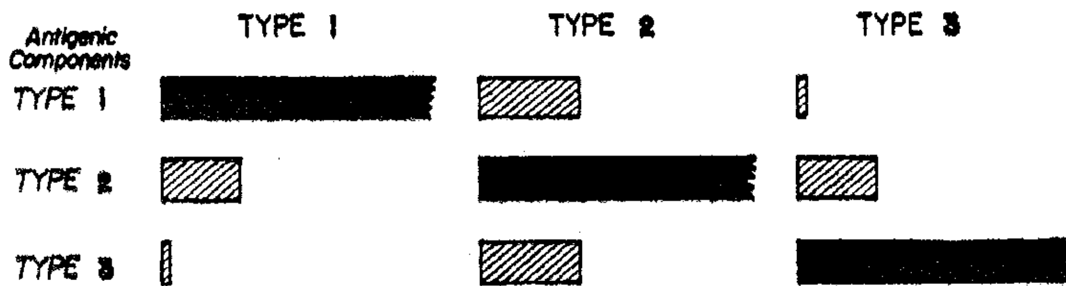


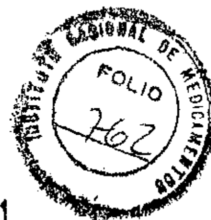
FIGURE 8. Data derived from vaccination studies. Interpretation suggesting the existence of major and minor antigens in strains of naturally occurring poliomyelitis virus.

similar, but it appears that the amounts are less than the amount of type 1 antigen present in the type 2 virus.

The Significance of the Relative Frequency of Paralytic Infections Due to the Three Virus Types

The foregoing observations led to a further inquiry into the possible role of the occult immunologic effects of a prior infection upon the outcome of a subsequent encounter with a heterotypic poliomyelitis virus under natural circumstances. The search for an answer to this question was made through analyses of data obtained in the course of other studies.

The purpose of the other studies to which I refer was to determine the frequency with which types 1, 2, and 3 infections occur under natural circum-



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who possess antibody only for a single type, 42 have type 1 antibody only, 25 have type 2 antibody only, and 33 have type 3 antibody only. This is in striking contrast to the far greater predominance of persons with only type 1 antibody in the paralyzed group. In the paralyzed group, and among those with antibody for one type only, 77 per cent had antibody for type 1, 11 per cent for type 2, and 12 per cent for type 3. Thus, it appears that the frequency with which antibody for only one of the respective types is found in paralyzed persons is about the same as is the frequency with which the respective types of virus have been isolated from paralytic cases that have occurred in the past several years. The fact that paralysis due to the type 1 virus occurs about seven times more frequently than does paralysis due to types 2 or 3 seems to be a function not of the frequency of distribution of these viruses in nature, but rather of the greater paralytogenic potential of the type 1 virus. It appears, too, that there may be a slight difference between types 2 and 3, with the type 3 virus being more innocuous in this respect.

Data on the frequency of occurrence of antibody for two virus types, in different combinations, among nonparalyzed and paralyzed persons is shown in FIGURE 10. It is clear that in both the nonparalyzed and in the paralyzed, combinations of types 1 + 3 antibody occurred more frequently than did combinations of types 1 + 2 or combinations of types 2 + 3. The combination 1 + 3, however, occurred more frequently in the paralyzed group than in the nonparalyzed, while the combination 1 + 2 and 2 + 3 occurred less frequently in the paralyzed than in the nonparalyzed. The greater frequency, in the paralyzed group, of individuals who have only type 1 antibody suggests that the type 1 virus is most likely to have been the reason for paralysis when the first encounter was with this virus. It is conceivable, therefore, that when the type 1 virus is one of the two viruses for which antibody was present, it, too, was the more likely cause of paralysis. That this is merely a statement of *probability* and not *always* true is, of course, indicated by the fact that some individuals, within the paralyzed population, have type 2 or 3 antibody only, or the combination 2 + 3. From these comparisons of the frequency of occurrence of the combinations of antibody for types 1 + 3, 1 + 2, and 2 + 3 in paralyzed and nonparalyzed individuals, it would seem that a prior type 2 infection must have a different meaning than does a prior type 3 infection, in terms of whether or not paralysis will ensue upon subsequent contact with the type 1 virus. From these data, it would appear that the experience of the previous type 2 infection reduced the probability that the subsequent type 1 infection would be a paralyzing experience or, alternatively, that the prior type 3 infection did not influence significantly—or if it did so, certainly to a much less degree—the probability of paralysis ensuing upon subsequent contact with the type 1 virus.

An analysis in further support of this interpretation is presented in TABLE 2, which shows that the probability of experiencing a nonparalytic infection due to type 1, 2, or 3 virus is essentially the same upon first or second exposure to a poliomyelitis virus. This is not the case in paralyzed individuals.

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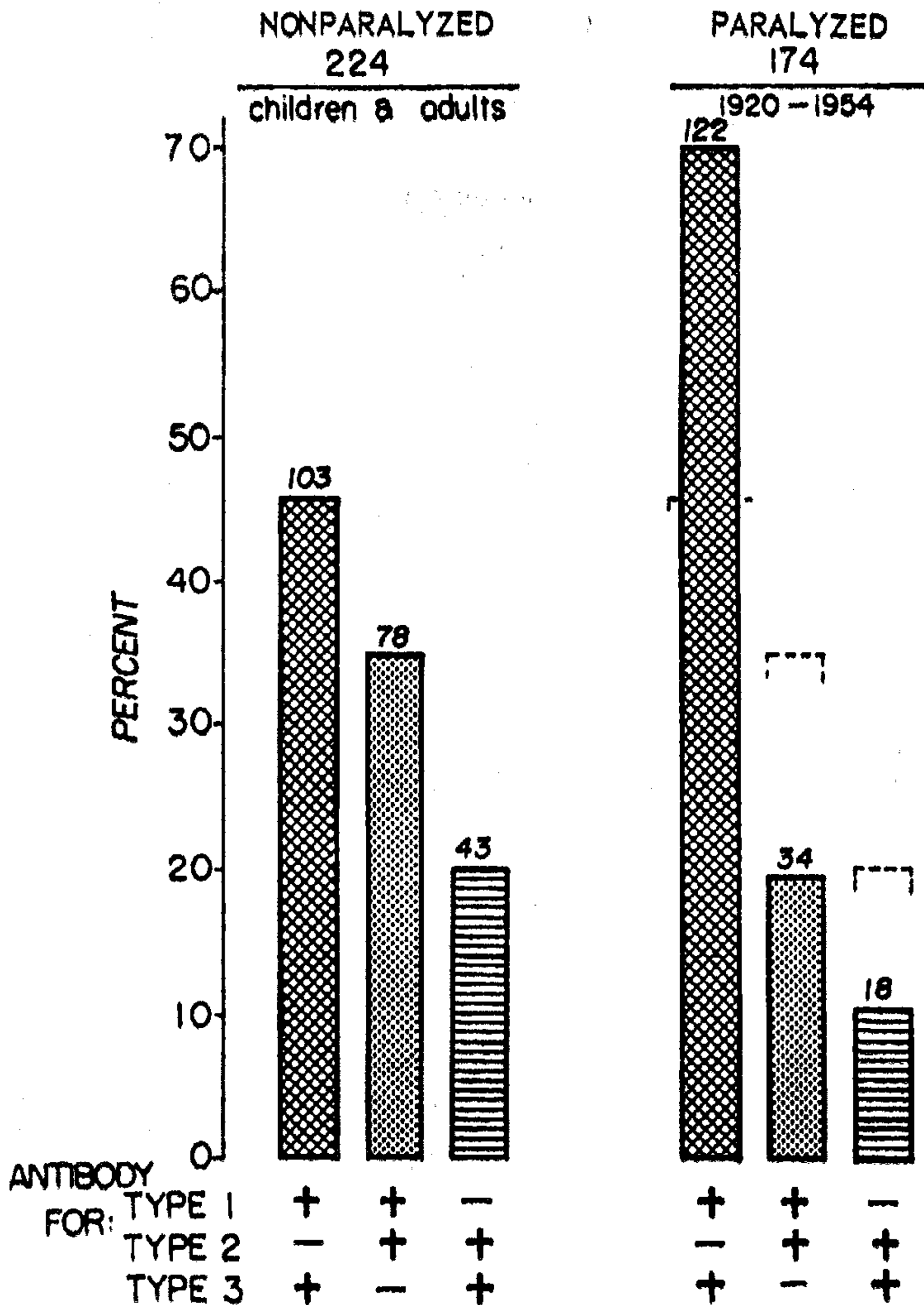

FIGURE 10. Relative frequency of occurrence of combinations of antibody for two types of poliomyelitis virus in nonparalyzed and paralyzed individuals.

A Concept of the Immune Mechanism in Poliomyelitis

If the observations presented in the two previous sections of this paper are linked in a relationship of cause and effect, then these two observations—namely (1) the evidence for the sensitizing effect of a prior type 2 infection,

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TABLE 2

FREQUENCY OF OCCURRENCE OF SERUM ANTIBODY FOR EACH TYPE OF POLIOMYELITIS VIRUS

Demonstrable antibody for	Nonparalyzed			Paralyzed		
	1	2	3	1	2	3
One type n.p.—349 p.—331	42%	25%	33%	80%	10%	10%
Two types n.p.—448 p.—380	40%	27%	33%	45%	15%	40%

N.p.—nonparalytic
p.—paralytic

as revealed by the response to the type 1 virus in a killed vaccine, and (2) the apparent influence of a prior type 2 infection in reducing the probability of the occurrence of paralysis upon subsequent contact with a type 1 virus—make it appear that the presence of a demonstrable level of circulating antibody for the type 1 virus was not necessarily required for the prevention of paralysis. It would appear that paralysis may have been prevented because of the existence of a hyperreactive immunologic mechanism engendered by the type 1 antigen present in the type 2 virus that had caused the first infection. If this be so, then it would appear that *the* prerequisite for persistence of immunity may not necessarily be the continued presence of antibody in the blood stream but rather a persistence of the state of immunologic hyperreactivity.

The observations presented bear not only on the practical problem of immunization of man against this disease but on many aspects of the theoretical question of the dynamics of the immune process. If we understood fully the dynamics of immunity in this disease then, theoretically, we could, by artificial means, reproduce the sequence of events that will lead to the immune state.

Many assumptions have been made in support of the selection of one approach or another to the solution of the practical problem. Those who think that only a live-virus vaccine will produce the necessary changes required for lifelong immunity must believe that the infectious process contributes something that cannot be conferred by any other form of antigenic stimulation. Then there are those who, whether they think that a live- or a killed-virus vaccine is necessary, are of the opinion that the actual presence of antibody in the circulating blood provides the *sine qua non* for effective immunity. It would follow that the required level of antibody in the blood would depend upon whether pathogenesis is *via* the blood stream or along nerve pathways. If the latter, then a higher level of antibody would be required than if the virus is always transmitted to the central nervous system (CNS) *via* the blood stream. But then, the possibility must be considered that the blocking effect against virus invasion of the CNS could conceivably take place before entrance of virus into the blood stream, and that this could be effected by a hyperreactive immunologic state which would be triggered by virus as it multiplies at the primary site of infection and, thereby, result in the prompt production of immune substances. If the hyperreactive state is adequate to produce a

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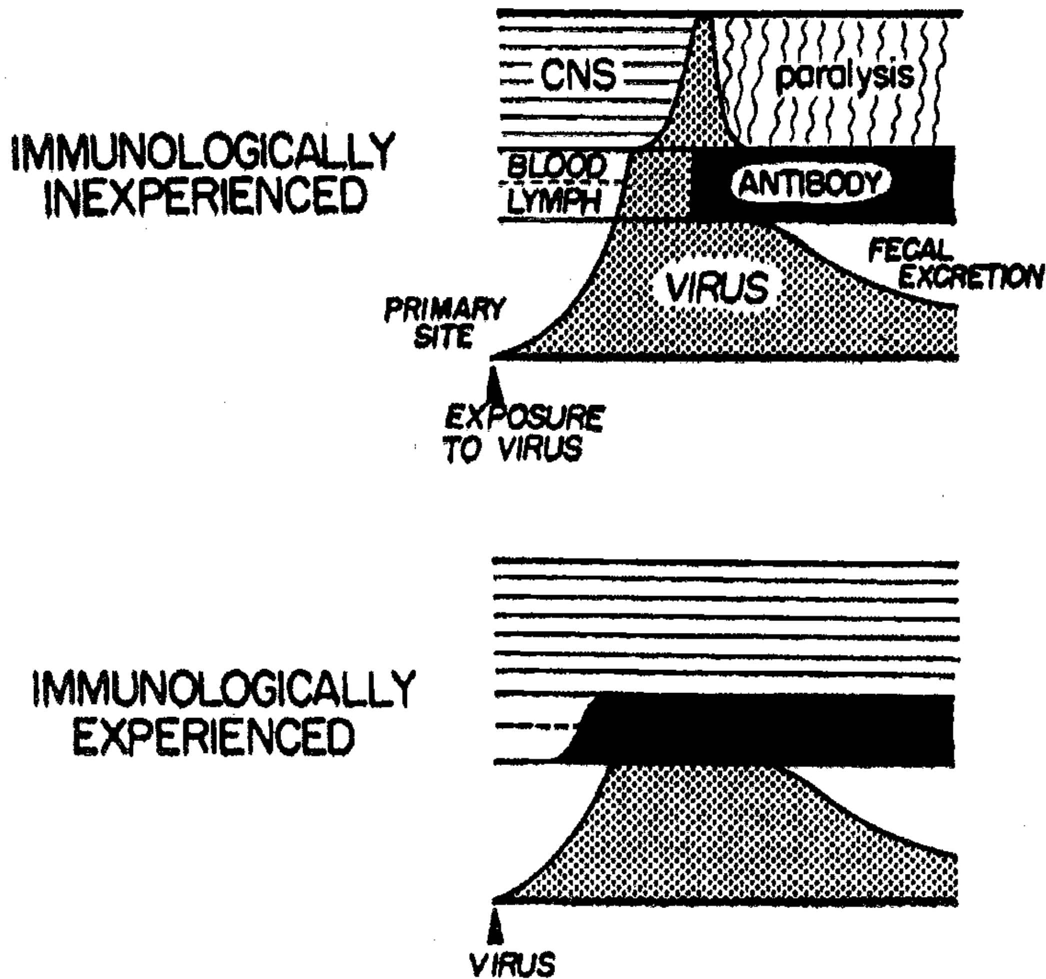

FIGURE 11. Concept of the influence of previous immunologic experience in the prevention of paralysis.

sufficient concentration of specific antibody, and if this effect would occur prior to the end of the multiplication period when the virus might normally enter the blood stream, then one can visualize a situation in which a state of immunity would exist even though there is no demonstrable antibody in the circulating blood at the time of virus exposure (FIGURE 11). Such immunity, however, would be dependent upon the capacity of the organism to respond to infection with the immediate production of antibody, sufficient either to prevent further multiplication, or to reduce the length of the period of active infection. The quantitative relationship between the rate of virus multiplication and the rate of antibody formation would determine the extent to which this barrier was effective. A highly reactive immune mechanism might be able to block the most actively multiplying virus, whereas a less reactive immune mechanism could conceivably be ineffective. Therefore, we must consider the *quantitative* concept of level of antibody required to prevent central nervous system invasion, and the *quantitative* concept as applied to measurements of degree of sensitization that would be required for immunity to be effective even in the absence of a measurable amount of circulating antibody.

The data presented suggest that a naturally acquired type 2 infection sensitizes the immune mechanism to a subsequent contact with a type 1 virus. It

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TABLE 3

SEROLOGIC RESPONSE TO NATURAL INFECTION IN VACCINATED CHILDREN

Subject No.	Age	Prevaccination May 4, 1954			10 Days after 3rd dose June 18, 1954			5 Months later Nov. 19, 1954		
		Type 1			Type 2			Type 3		
		May	June	Nov.	May	June	Nov.	May	June	Nov.
C-7930	5	0	16	8	0	128	8192+	0	32	4
C-7948	7	0	16	16	0	128	8192+	0	1024	128
C-8033	12	0	16	8	0	8	8192+	0	128	32
C-7983	8	0	4	1024	0	4	32	0	128	32
C-7957	6	0	32	2048	0	32	128	256	8192+	8192+

Vaccinated on May 4, 1954; blood tested 10 days after third dose, June 18, and five months later, November 19.

TABLE 4

TWO-YEAR FOLLOW-UP IN FIRST GROUP OF SUBJECTS IN WHOM VACCINATION STUDIES WERE UNDERTAKEN

Age at time of first vacc.	Identification No.	Type 2 antibody titer vs. 100 ID_{50} virus			
		Before vacc.*	2 Mos.	Booster at 20 mos.†	22 Mos.
years					
2	W-44	0	32	8	2048
4	W-31	0	64	8	1024
5	W-18	0	16	8	512
6	W-27	0	8	2	512
8	W-20	0	128	64	256
9	W-33	0	8	0	256
10	W-8	0	4	0	512
10	W-26	0	16	8	256
11	W-74	0	32	4	512
14	W-34	0	16	8	256
17	W-1	0	32	16	128
17	W-32	0	32	16	N.T.‡
31	W-3	0	16	8	256

* 2 Doses I.D. of 0.1 ml. each 6 weeks apart monovalent MEF1 1:250 HCO—1° C.
† 1 Dose I.M. of 1 ml. trivalent vaccine.
‡ Not tested.

would follow from this that the amount of type 1 antigen required to produce the sensitizing effect must be very small indeed. Since a measurable amount of type 1 antibody either does not develop, or does not persist for any length of time after a type 2 infection, it would follow that the resistance to the development of type 1 paralysis, which is engendered by the prior type 2 infection, is mediated by some means other than type 1 antibody present in the circulating blood *at the time of exposure* to the type 1 virus. On the assumption that antibody is the agent that effects immunity, this must mean, then, that type 1 antibody is produced sufficiently rapidly after exposure, and in sufficient concentration, to exert a blocking effect upon virus produced at the primary site of infection.

According to this concept, a critical index of immunity would be provided by

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a test for the presence of the hyperreactive state rather than by a test that would determine the presence or absence of antibody in the serum. Even if an individual does not possess circulating antibody, he might well be immune if he does possess a hyperreactive immune mechanism. Therefore, an indicator of the degree of duration of *persistence* of immunity following vaccination would be provided by testing for the degree of hyperreactivity at intervals after vaccination. If the hyperreactive state persists either for long periods, or throughout life, then it is conceivable that immunity to the development of paralytic poliomyelitis would persist similarly (TABLES 3 and 4).

Although the presence of unequivocal concentrations of antibody in the circulating blood is a good indicator of the existence of a hyperreactive immunologic state (TABLE 4), it is suggested that the hyperreactive mechanism, even in the absence of demonstrable circulating antibody, is the minimum requirement for effective immunity. For this reason, tests are being devised to measure degree of immunologic hyperreactivity. Such a test could be based upon the booster phenomenon, since the persistence of the hyperreactive state is reflected in the booster response. Thus, the booster phenomenon would be useful not only to reinforce the level of circulating antibody, but as a test that would reflect the degree of persistence of immunologic hyperreactivity.

Discussion of the Paper

DOCTOR ALBERT MILZER (*Department of Microbiology, Michael Reese Hospital, Chicago, Ill.*): Doctor Salk's results parallel those which we have obtained with a noninfectious vaccine similar to his but inactivated by ultraviolet irradiation[1] instead of formalin. I have been asked to present recent results obtained by our group* in the immunization of children in Morgan County, Illinois, with an aqueous trivalent tissue culture poliomyelitis vaccine inactivated by ultraviolet irradiation. The vaccine used in these studies was prepared as described previously,[1] but no preservative was added because we found that the irradiated vaccine containing no preservative still showed antigenicity in animal potency tests after six months' storage in the refrigerator. The strains of virus selected to represent the three types of poliomyelitis were the same as those used previously, except that the Brunhilde strain was substituted for the Mahoney strain for type 1. The MEF_1 strain was used for type 2, and the Saukett strain for type 3.

One hundred and twenty normal children ranging in age from two to seven years were given three injections of the irradiated vaccine during the late winter and spring of 1954. All of the children were bled prior to inoculation and received two injections intramuscularly one week apart. The third inoculation was given three to four weeks after the second. All the children were bled two weeks after the third inoculation. Antibody titrations were carried out in roller-tube cultures of trypsinated monkey kidney tissue cells. Since we were interested in determining whether primary immunization could be stimulated by the irradiated vaccine, all prevaccination serum antibody titra-

* Including the late Doctor S. O. Levinson of the Michael Reese Research Foundation, Chicago, Ill.; Doctor H. J. Shaughnessy, Doctor Ruth Church, and Doctor Leonard Schuman of the Illinois Department of Public Health; Doctor A. M. Wolf, Doctor F. Oppenheimer, and Martha Janota of the Michael Reese Research Foundation.

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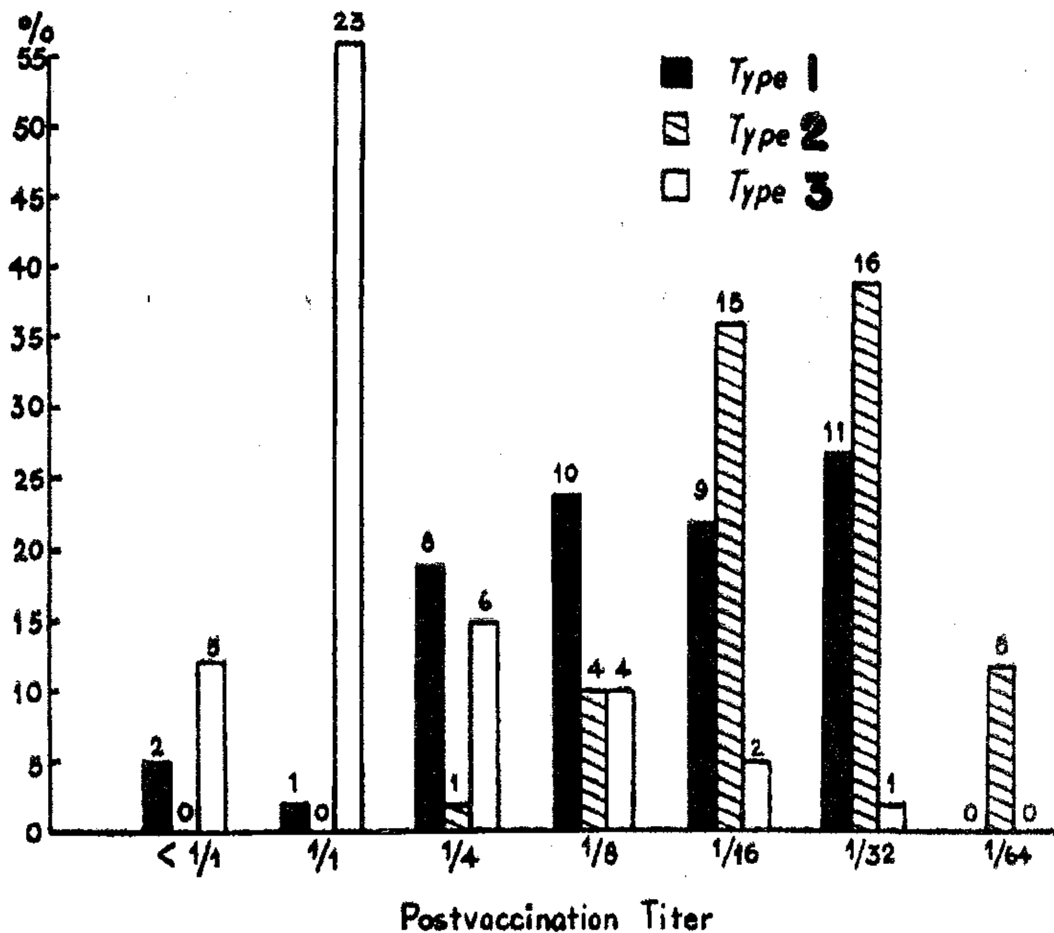

FIGURE 1. Antibody response to three types of poliomyelitis virus in 41 children with no detectable polio antibody prior to vaccination with aqueous poliomyelitis trivalent tissue culture irradiated vaccine.

tion were carried out initially with 10 ID_{50} of each of the 3 virus type components in neutralization tests with undiluted serum (1/1), including serial twofold dilutions starting with 1/4. It was found that 41 children had no detectable antibodies (<1/1) to 10 ID_{50} of the three virus types. Results obtained are shown in FIGURE 1. Thirty-eight or 92 per cent developed a postvaccination antibody titer of 1/4 or greater to type 1 virus; 41 or 100 per cent to type 2 virus, and 13 or 31 per cent to type 3 virus. The majority of the remaining 79 children who had a prevaccination serum titer of 1/1 or greater to 10 ID_{50} to one or more of the 3 virus type components subsequently developed a significant (fourfold or greater) antibody response in their postvaccination serums to 100 ID_{50} of the 3 virus types.

No significant local or generalized reactions were encountered in the 120 children vaccinated with the irradiated vaccine. Urinalysis of all of the children was normal when tested at the time of the last blood specimen. Furthermore, we were unable to detect Rh or Hr antibodies or sensitization in 32 Rh negative children in our series of immunized children.

We have found a correlation between antibody response to the irradiated vaccine in man, the mouse,[2] the monkey, and the rabbit.[3] More recently, we have prepared an improved irradiated vaccine from tissue culture virus sus-

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pensions with ID_{50} titers of 10^{-7} to 10^{-8} compared to earlier relatively low titered preparations and have also made modifications in the technique of inactivation. These improvements in vaccine preparation are reflected in superior antibody responses that have been obtained recently in both immunized mice and monkeys.

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ANTIGEN CONTENT OF INACTIVATED POLIOVIRUS VACCINE FOR USE IN A ONE- OR TWO-DOSE REGIMEN *

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ABSTRACT

The immunologic response to inactivated poliovirus vaccine following one and two doses has been studied in infants in developing and developed countries using vaccine prepared at the Rijks Instituut voor de Volksgezondheit, The Netherlands. Virus was grown in microcarrier cultures of monkey kidney cells, purified, concentrated, and inactivated with formalin. The vaccines used contained different quantities of D-antigen units for each of the three types. The data reveal that both antibody and immunologic memory (booster-type responsiveness) were induced in virtually all individuals following a single dose of a sufficient quantity of antigen. Immunologic memory was readily revealed by the booster-type response following a second dose given six months after the first. The degree of booster-type response to a second dose is linked primarily to the quantity of antigen used for primary immunization, and secondarily to the quantity of antigen used for the booster dose.

The data base is presented for formulating the antigen content of an inactivated poliovirus vaccine that can be relied upon to be protective after the first dose when given alone or when incorporated with combinations of other antigens (diphtheria-pertussis-tetanus) that may require two or more doses.

KEY WORDS: INACTIVATED POLIOVIRUS VACCINE; ANTIGEN CONTENT

There is a need, especially in developing countries, for a vaccine against poliomyelitis that can be relied upon to be protective after the first dose, either when given alone or when combined with other antigens (e.g., DPT) that may require two or more doses (1). For the purpose of establishing the quantity of inactivated poliovirus antigens for a trivalent vaccine that would produce such an effect, a series of serological studies in infants was initiated in 1977 in developing and developed countries (2).

Experimental inactivated poliovirus vaccines (IPV) for this purpose containing different quantities of poliovirus D-antigen units for each of the three types, were prepared at the Rijks Instituut voor de Volksgezondheid (RIV), The Netherlands, using virus propagated in microcarrier cultures of monkey kidney cells, purified, concentrated, and inactivated with formalin (3). In some of the studies vaccines prepared in other laboratories were included for comparison.

A summary of the results of the first study (2), which was carried out in Mali in 1977—78, is shown in Fig. 1. This figure reveals the percent of infants (4—7 months of age, in groups of 25—34), with antibody titers of 1 : 4 or greater one month following a single dose of vaccines containing the different quantities of D-antigen units indicated on the abscissa, graded in four-fold steps (i.e., 320 to 5

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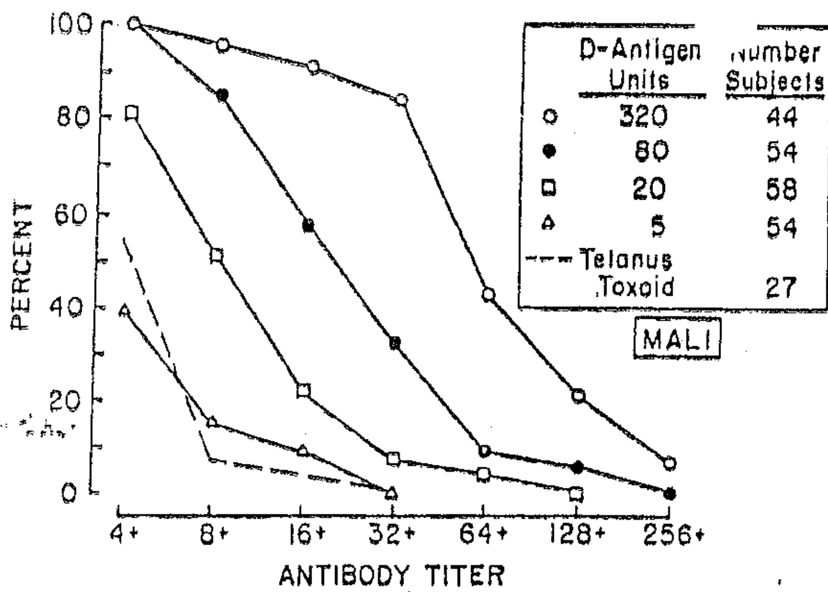


Fig. 2. Mali inactivated poliovirus vaccine study. Cumulative percent distribution of type 1 poliovirus antibody titers one month after one dose of vaccines with different D-antigen unit content per dose. Vaccines prepared by Rijks Instituut voor de Volksgezondheid. (Reprinted courtesy of S. Karger, Basel) (4).

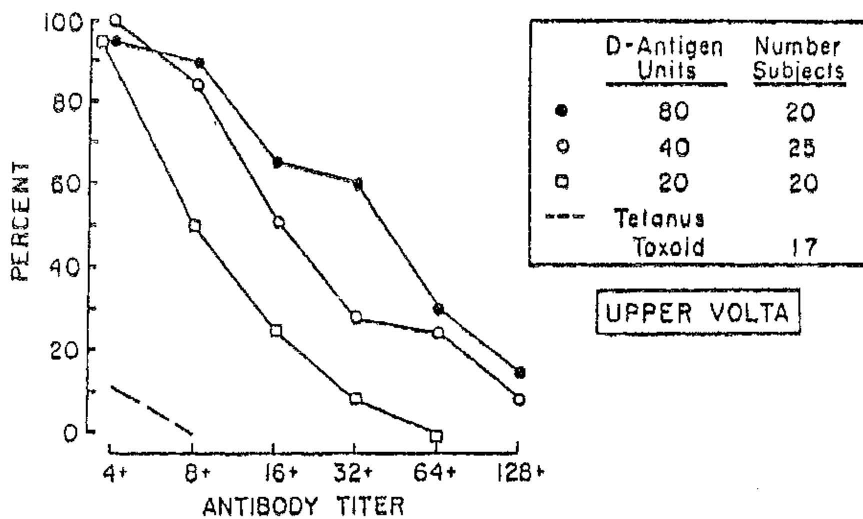
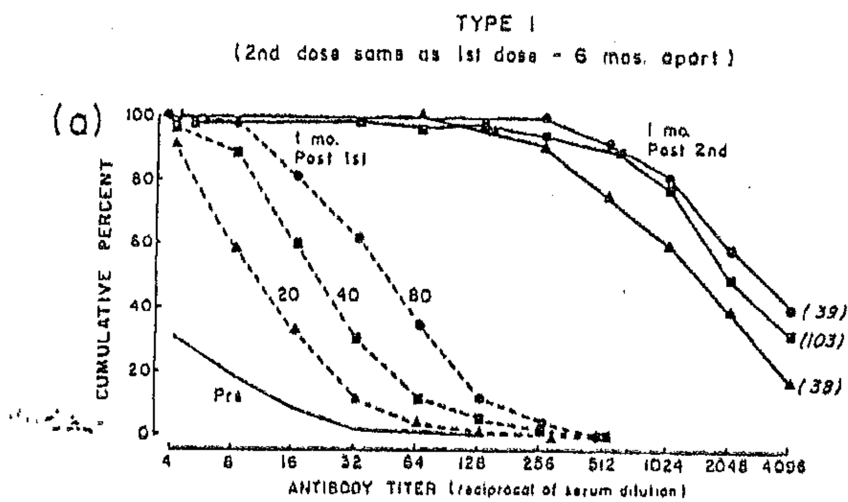


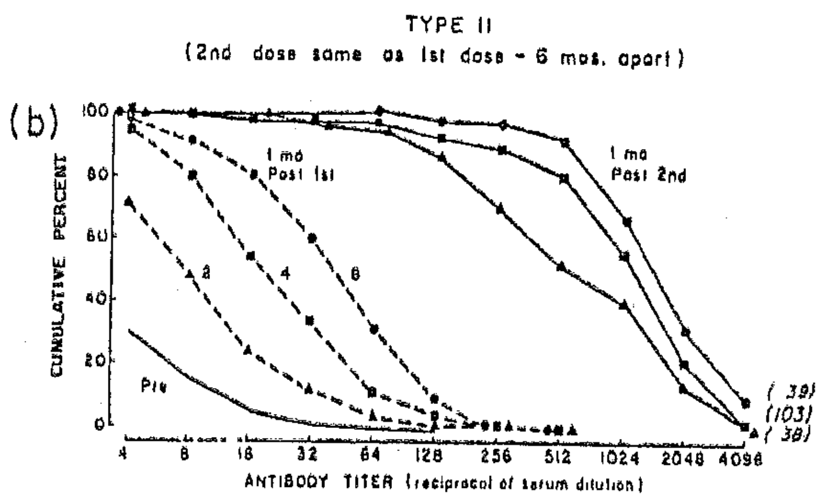
Fig. 3. Upper Volta inactivated poliovirus vaccine study. Cumulative percent distribution of type 1 poliovirus antibody titers one month after one dose of vaccines with different D-antigen unit content per dose. Vaccines prepared by Rijks Instituut voor de Volksgezondheid. (Reprinted courtesy of S. Karger, Basel) (4).

group was divided into two parts. The first (approximately 50—60 %) received a uniform dose of the RIV vaccine containing 40-4-16 D-antigen units for types 1, 2, and 3, respectively. The second group received the same vaccine for the second dose as for the first. In all groups a blood sample was collected one month after the second dose.

Fig. 4a shows the distribution of type 1 antibody prevaccination, one month post first, and one month post the second dose in the groups given the three different RIV vaccines containing 80, 40, and 20 D-antigen units, respectively, for both the first and the second doses.

It is clear that more than 90 % have detectable antibody to type 1 after the first dose and the levels of antibody attained are in proportion to the D-antigen content of the vaccines. It is also clear that after the second dose a striking booster effect is observed. The same pattern is seen for type 2 in Fig. 4b





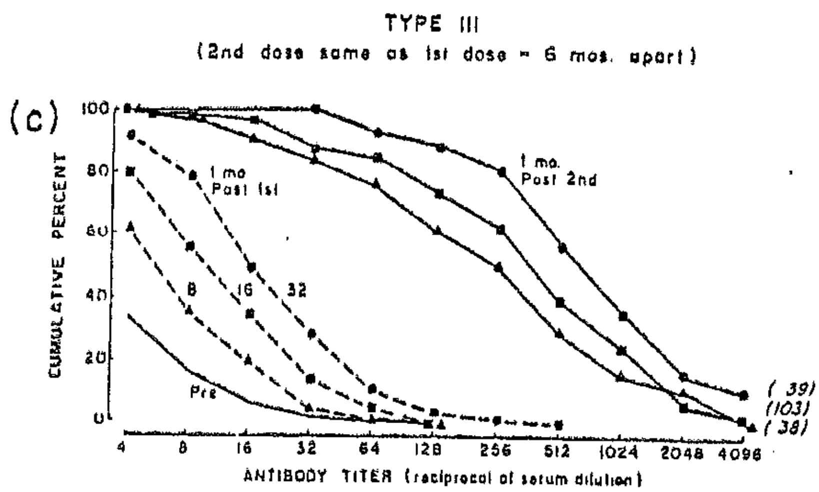


Fig. 4. Finland inactivated poliovirus vaccine study. Cumulative distribution of polio antibody titers one month after a first dose and one month after a second dose (administered six months after the first dose) of vaccines containing: (a), 20, 40, or 80 D-antigen units for type 1; (b) 2, 4, or 8 D-antigen units for type 2; and (c) 8, 16, or 32 D-antigen units for type 3. Vaccines prepared by Rijks Instituut voor de Volksgezondheid.

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FIRST DOSE VACCINE	D-ANTIGEN UNITS	PRE BOOSTER	POST BOOSTER	BOOSTER DOSE VACCINE	D-ANTIGEN UNITS	...BER ...JECTS
RIJKS	80	●- - -●	●——●	RIJKS	40	52
RIJKS	40	□- - -□	□——□	"	"	46
RIJKS	20	○- - -○	○——○	"	"	63
MFG B	36	▲·····▲	▲— —▲	"	"	42
MFG C	30	△·····△	△— —△	"	"	45

FINLAND

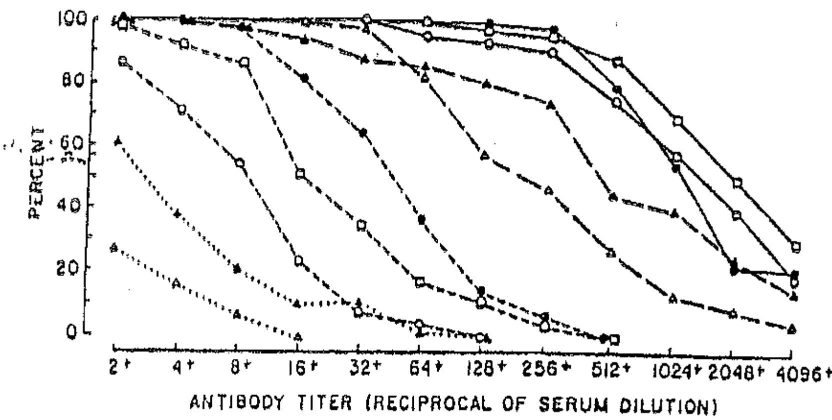

Fig. 6. Finland inactivated poliovirus vaccine study. Cumulative percent distribution of type 1 poliovirus antibody titers before and after a booster dose administered six months after a first dose of Rijks Instituut voor de Volksgezondheid (RIJKS) vaccines and manufacturers (MFG) B and C vaccines. (Reprinted courtesy of S. Karger, Basel) (4)

TYPE I

(2nd dose; RIV 40 D-antigen units - 6 mos. post 1st)

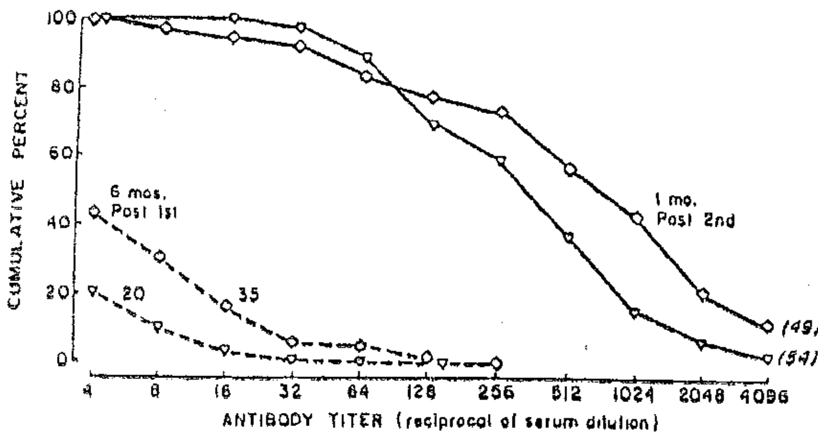

Fig. 7. Finland inactivated poliovirus vaccine study. Cumulative percent distribution of type 1 poliovirus antibody titers six months after a first dose of two conventional vaccines (containing 20 and 35 D-antigen units per dose) currently used in Finland, and one month after a second dose of Rijks Instituut voor de Volksgezondheid (RIV) vaccine (administered six months after the first dose) containing 40 D-antigen units ped dose. Numbers in parentheses = number of subjects per group.

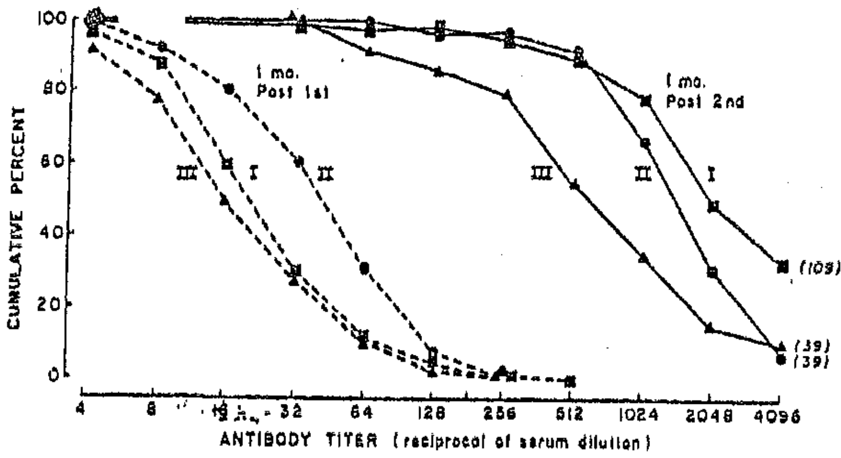

Fig. 10. Finland inactivated poliovirus vaccine (IPV) study: types 1, 2, and 3 antibody levels after a first and second dose (administered six months apart) of proposed IPV for general use (40-8-32 D-antigen units per dose for types 1-2-3, respectively). Vaccine prepared by Rijks Instituut voor de Volksgezondheid. Numbers in parentheses = number of subjects per group. (Reprinted courtesy of Behring Institute Mitteilung, Marburg) (5)

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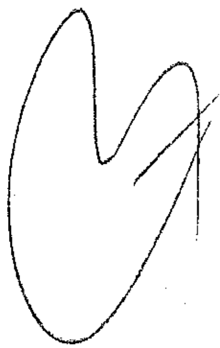

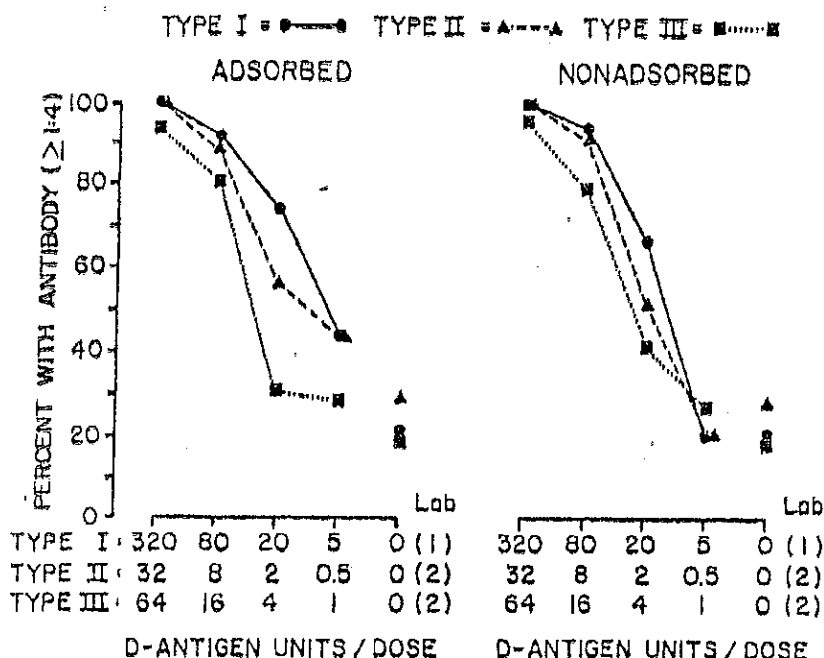

Fig. 1. Relationship between D-antigen units and antibody response to a single dose of inactivated poliovirus vaccine: composite of selected results from two laboratories. (Reprinted courtesy of S. Karger, Basel) (2).

type 1 D-antigen units per dose, 32 to 0.5 type 2, and 64 to 1 type 3). The data reveal an essentially linear relationship between the amount of antigen contained in the vaccine and percent seropositivity following a single dose. The frequency with which residual maternal antibody or antibody acquired by natural infection was observed is indicated by the points for the control group shown on the right of the chart.

Fig. 2 reveals the percent distribution of type 1 poliovirus antibody titers one month following a single dose of the vaccines used in the Mali study. By comparison with a group given tetanus toxoid for control, the dosage response effect is revealed by the relative positions of the distribution curves in the groups given 320, 80, 20, and 5 D-antigen units for type 1, respectively.

In view of these results a further study was carried out in Upper Volta in 1978—79 (4) using two-fold differences in antigen concentration over the midportion of the range studied in Mali. Fig. 3 shows the response to the type 1 component after a single dose of 80, 40, and 20 D-antigen units. These data reveal the presence, one month later, of antibody titers of 1 : 4 or greater in more than 90 % of vaccinees, as compared to about 10 % in the control group given tetanus toxoid; the differences seen in the relative position of the antibody titer distribution curves reflect the differences in the quantity of antigen contained in each of the three vaccines tested in this study.

A second dose was given six months later in accordance with the protocol for this study. While persistence of antibody was demonstrated after six months and a sharp booster response observed, the prevalence of natural infections in Mali and Upper Volta interfered with the interpretation of the data on dose response effects and antibody persistence. To avoid this complication a further investigation was carried out in Finland and in Sweden, since both are free of naturally occurring poliomyelitis virus infections and in both only IPVs have been in continuous use. The data to be reported at this time are only from the Finland study (4).

The basic design of the Finland study is shown in Table 1. Five vaccine preparations were used. Three were prepared at the RIV; and one each was from two different manufacturers of IPV, one of these (manufacturer C) is the vaccine in general use in Finland. Approximately 100 infants were in each group and received their first dose at five months of age. A blood sample was collected by venipuncture prior to the first dose, one month later, and again six months later just prior to a booster dose. For the booster the

T[illegible] 1

Poliovirus vaccines studied in Finland (2 doses at 6-month interval).

D-antigen units for types 1-2-3		
Part 1		Part 2
First dose	Booster dose	First and booster doses
Rijks Institute: 80-8-32, 40-4-16, 20-2-8	Rijks Institute: 40-4-16, 40-4-16, 40-4-16	Rijks Institute: 80-8-32, 40-4-16, 20-2-8
Manufacturer B: 35-1.5-7	40-4-16	Manufacturer B: 35-1.5-7
Manufacturer C: 20-2.2-4	40-4-16	Manufacturer C: 20-2.2-4

and for type 3 in Fig. 4c. Although the antibody response to the first dose is not as uniformly as high as for types 1 and 2, nevertheless, after the second dose all responded with antibody levels of 1 : 4 or greater and more than 90 % with antibody levels of 1 : 64 or greater.

The degree of persistence of type 2 antibody six months after the first dose is indicated in Fig. 5a. This figure also reveals antibody levels one month after the second dose following a uniform second dose consisting of 40 D-antigen units of the RIV vaccine. Similar persistence was observed for type 2 antibody as seen in Fig. 5b. Fig. 5c indicates a distinct decline in type 3 antibody levels in the six months post first dose in contrast to the constancy observed for types 1 and 2. For all three types there is a tendency for the differences observed post first dose to be reduced after the second dose.

Fig. 6 shows the antibody distribution pre and post booster dose comparing the RIV vaccines and those of manufacturers B and C; the type 1 D-antigen components of the latter correspond to that of the RIV 40 and 20 D-antigen unit vaccines. However the type 1 primary antibody response to the manufacturers' vaccines was distinctly less than to the RIV vaccines; there was also a lesser response to a uniform booster dose. An explanation is proposed below for the dissociation seen here between D-antigen unit content and antigenic potency in the RIV and the other vaccines. This figure also reveals that the antigenic potency of the vaccine used for the primary dose influences the degree of antibody response to a booster dose.

Fig. 7 shows the superior ef[illegible] of higher potency vaccine used for the booster dose when low potency vaccine is used for primary immunization, as compared to Fig. 8 which shows the inferior effect when the same low potency vaccine is used for both booster and primary doses.

Fig. 9 shows more clearly the dissociation between D-antigen unit measurement and antibody response in humans for the type 1 component of the commercial vaccines when compared with the performance of the vaccines prepared by the RIV methods. It is believed that these differences are attributable to technical factors. The RIV vaccines are made from virus purified and concentrated before inactivation with formalin; however, vaccines prepared by manufacturers B and C are inactivated with formalin and either not further treated or are purified and concentrated thereafter. This question is currently under investigation. The shaded area of Fig. 9 shows the geometric mean antibody response to a single dose of IPV containing the 40-8-32 D-antigen unit formula which is now under study in several countries.

Fig. 10 shows the antibody distribution curves post first and post second dose for types 1, 2, and 3 of the RIV 40-8-32 D-antigen unit vaccine. It is to be noted that the IPV used in The Netherlands for many years has contained the 20-2-7.5 D-antigen units, respectively, in association with diphteria-pertussis-tetanus antigens (DPT).

By comparison with the vaccines currently used in Finland (Fig. 8), where poliomyelitis has been brought under complete control and where virus has been eliminated from circulation, the more potent vaccine referred to in Fig. 10 can be expected to be protective after one or two doses.

A detailed report will be made at a later time of early favorable observations in progressively expanding field studies in endemic areas in West Africa where such poliovirus vaccines compined with DPT are being used in routine immunization programs — which also include immunization against BCG, measles and yellow fever (6).

Further and more extensive studies are now being planned using IPV of the potency here proposed, prepared according to the RIV methods. In a more recent development, the use of an approved continuously propagating cell line has eliminated the need for monkeys

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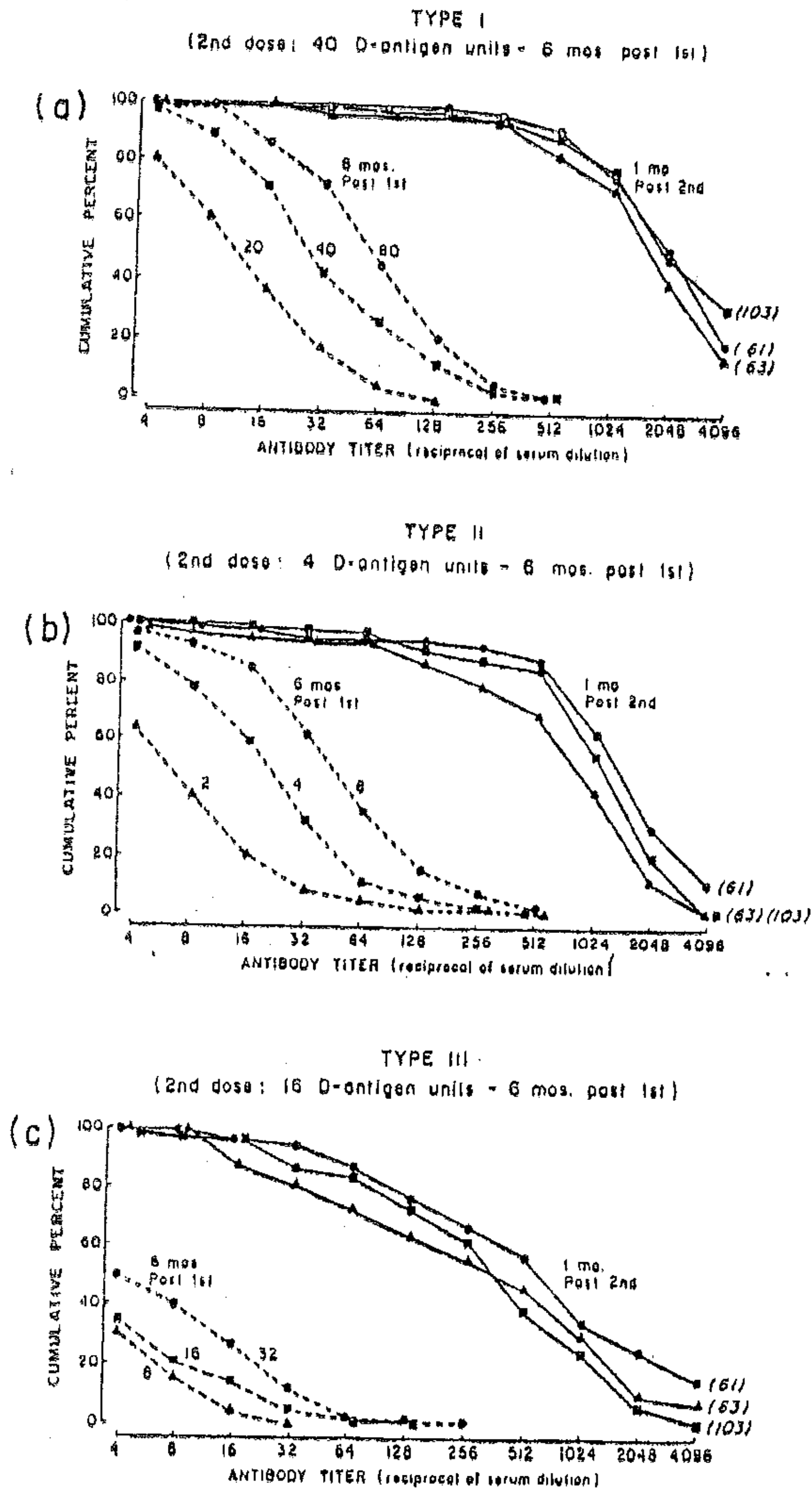

Fig. 5. Finland inactivated poliovirus vaccine study. Cumulative percent distribution of poliovirus antibody titers six months after a first dose and one month after a second dose (administered six months after the first dose) of vaccines containing: (a) type 1 — 20, 40, or 80 D-antigen units for the first dose, and 40 D-antigen units for the second dose; (b) type 2 — 2, 4, or 8 D-antigen units for the first dose, and 4 D-antigen units for the second dose (c) type 3 — 8, 16, or 32 D-antigen units for the first dose, and 16 D-antigen units for the second dose. Vaccines prepared by Rijks Instituut voor de Volksgezondheid. Numbers in parentheses = number of subjects per group.

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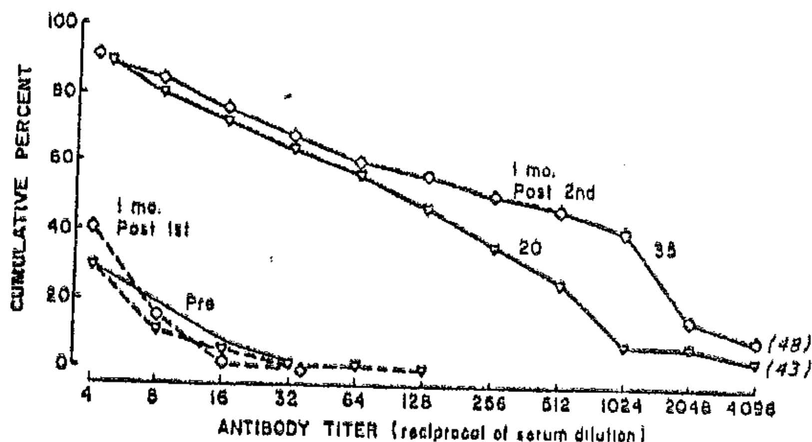

Fig. 8. Finland inactivated poliovirus vaccine study. Cumulative percent distribution of Type 1 poliovirus antibody titers one month after a first dose and one month after a second dose (administered six months after the first dose) of two conventional vaccines (containing 20 and 35 D-antigen units per dose) currently used in Finland. Numbers in parentheses = number of subjects per group.

..o provide cells as substrate for producing virus (7). This now permits production of IPV on a very large scale thereby sharply reducing cost per dose to a level accessible to developing countries (8).

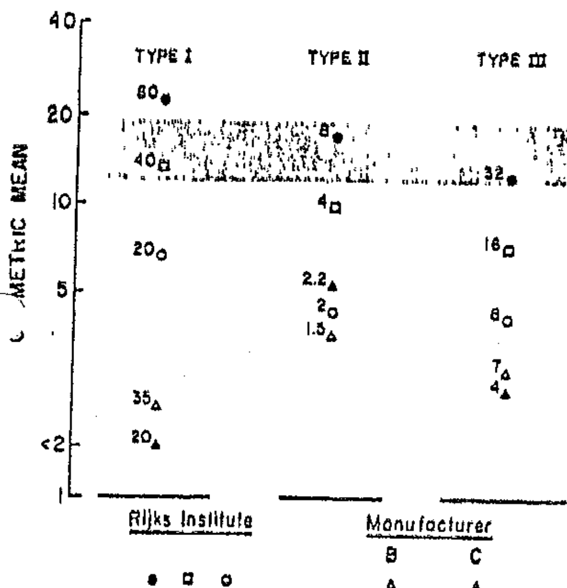

Fig. 9. Finland inactivated poliovirus vaccine study. Geometric mean antibody titers induced by a first dose of vaccines of different D-antigen unit content; comparison of vaccines prepared by Rijks Instituut voor de Volksgezondheid and by manufacturers B and C. (Reprinted courtesy of S. Karger, Basel) (4)

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One-Dose Immunization Against Poliomyelitis Using Killed Poliovirus Vaccine.

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Running head: One-dose immunization against polio

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ABSTRACT

Recent advances in the technology of vaccine manufacture and standardization allow production of large quantities of killed poliovirus vaccine of uniform potency. Vaccines produced by the Rijksinstituut voor Volksgezondheid en Milieuhygiene in The Netherlands were characterized in humans in a series of studies started in 1977. Monovalent pools of poliovirus types 1, 2, and 3 have been stored for use as reference standards. A single dose of vaccine that contains 40, 8, and 32 D-antigen units of types 1, 2, and 3 poliovirus, respectively, can be expected to induce life-long immunity to paralytic poliomyelitis when administered to six month old infants, and preliminary results indicate the same for two month olds. Studies are underway to determine the quantity of antigen needed to immunize newborn infants with a single dose.

Key words: poliomyelitis, poliovirus, poliovirus vaccine, immunization, immunization schedule, vaccination, viral vaccines

INTRODUCTION

During the poliomyelitis epidemic in the United States in 1959, there was an inverse linear relationship between the logarithm of the paralytic attack rate and the number of doses of killed poliovirus vaccine administered (1). This relationship suggested that the induction of immunity to paralytic poliomyelitis was a threshold phenomenon that resulted from a single immunizing event: immunity to paralysis was induced by the first dose or the second dose or the third dose, etc., not as a cumulative result of multiple doses.

On the average, killed poliovirus vaccine used in the United States from 1955 to 1959 was only 55 percent effective following each individual dose (1,2). Some lots of vaccine were of greater potency and vaccines used at the time in some other countries were 80 to 90 percent effective following each dose (1). It was apparent that the use of higher potency vaccines would be more efficient because it would reduce the number of doses needed to ensure immunity. In 1960 it was suggested that immunity to paralysis could be induced with a single dose of suitably potent killed poliovirus vaccine (1).

In the last twenty years advances have been made in methods of cell culture, virus purification and concentration, and vaccine standardization. Killed poliovirus vaccine of high potency can now be produced reliably in large quantities. In 1977 a series of studies was undertaken to establish the antigen content that would protect against paralytic poliomyelitis with a

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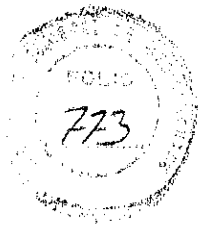
single dose.

The trivalent poliovirus vaccines studied were prepared in The Netherlands at the Rijksinstituut voor Volksgezondheid en Milieuhygiene (RIVM) from monovalent vaccine pools that have been stored for future use as reference standards (3). Studies were performed both in countries with warm climates where poliomyelitis is endemic and in countries with temperate climates where poliomyelitis has been controlled; the results of some of the earlier studies have been described previously (4-8). The purpose of this paper is to present all the results to date and to discuss strategies for use of high potency killed poliovirus vaccine. We report the characteristics of a vaccine that may be used as a reference standard, a vaccine that reliably induces immunity following a single dose.

CHARACTERIZATION OF REFERENCE VACCINE

The first study in this series was performed in Mali in 1977-1978 (4). Five vaccines were tested that contained different quantities of the three poliovirus types: figure 1 shows the response of infants to a single dose of these vaccines. There is a direct relationship between the antigen content of the vaccines and the percentage of infants with an antibody response ≥1:4 one month after vaccination. Similar results were obtained in Upper Volta in 1978-1979 using vaccines that differed in antigen concentration by two-fold steps over the mid-portion of the range studied in Mali (5).



In the Upper Volta study, a challenge dose of vaccine was also administered six months after the single primary dose of vaccine (5). The purpose of this challenge dose was to test for the presence of immunologic memory as revealed by a secondary-type antibody response. The prevalence of natural poliovirus infection in the population made the interpretation of these data difficult. Further investigations to characterize the reference vaccine were therefore carried out in Finland and Sweden where neither wild nor live-vaccine polioviruses are circulating. Both of these Scandinavian countries are free of domestically arising wild poliovirus infections and neither uses live, oral poliovirus vaccine (9).

In six month old infants in Finland, a direct relationship was again demonstrated between the antigen content of the vaccine and the antibody response following a single dose (fig. 2). The relationship is linear over the range studied. Two-fold differences in antigen content can be distinguished.

The response to challenge with vaccine is shown for type 1 poliovirus in figure 3; similar results were obtained for types 2 and 3 (6). The figure shows the percentage of infants with type 1 antibody titers at or above the indicated levels. As expected, antibody titers one month after immunization with one dose of vaccine are proportionate to the antigen content of the vaccine. Following a challenge dose of vaccine six months after immunization, all children had very high levels of antibody. This secondary-type response after antigenic challenge indicates

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that immunologic memory was present. The fact that even those children who had little or no detectable antibody following the first dose demonstrated a secondary-type response indicates that sensitization of the immune system can occur even in the absence of a detectable primary antibody response (10).

We studied the progressive development of immunologic memory in infants in Finland by administering a single immunizing dose of killed poliovirus vaccine followed by a single challenge dose of vaccine at different intervals: 1, 2, 4, 6, or 12 months later (fig. 4). By two months after immunization, all children respond to antigenic challenge with titers of 1:32 or greater. The magnitude of the secondary-type response increases until six months after primary immunization, after which no further augmentation is observed: geometric mean antibody titers one month after antigenic challenge were 115, 356, 803, 1219, and 1249 in the 1, 2, 4, 6, and 12 month-interval groups, respectively.

The effect of one- and two-dose primary immunization schedules was tested in Sweden. Infants were immunized starting at seven to nine months of age, either with a single dose of killed poliovirus vaccine or with two doses one month apart. A challenge dose of vaccine was administered six months after the first dose. All children in both groups had serum antibody titers ≥1:64 one month after antigenic challenge (fig. 5). One dose for primary immunization in this age group was sufficient for induction of immunologic memory in all children.

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The response of infants immunized starting at two months of age has been studied in Israel. In 1979-1980, a combined diphtheria-tetanus-pertussis-polio vaccine (DTP-polio) was tested that contained 40, 4, and 16 D-antigen units (DU) of poliovirus types 1, 2, and 3, respectively (7). One hundred fifteen infants were vaccinated at two and at three-and-a-half months of age. One month after the second dose, 100 percent of children had circulating antibody titers ≥1:10 for all three poliovirus types (geometric mean titers of 347, 158, and 413 for types 1, 2, and 3, respectively). Six months later approximately half of the children received a challenge dose of DTP-polio vaccine and all of them demonstrated a secondary-type response: the geometric mean antibody titers were 2285, 1701, and 1949 against poliovirus types 1, 2, and 3, respectively.

This group of children in Israel has been followed for two years after the challenge dose of vaccine (8). All of the 86 children tested have maintained antibody titers ≥1:10. Geometric mean titers declined three- to eight-fold during the two year follow-up period, but these titers (approximately 200, 100, and 340 for poliovirus types 1, 2, and 3, respectively) are approximately two- to five-fold greater than those among a parallel group of 53 children followed for two years after the last of five doses of oral live poliovirus vaccine that were administered during the first year of life (four doses of trivalent and one dose of monovalent type 1 vaccine).

In more recent trials in Israel, killed poliovirus vaccine has been used that contains 40, 8, and 32 DU of poliovirus types

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1, 2, and 3, respectively. This is twice the amount of types 2 and 3 antigen in the vaccine used in the earlier trials; as discussed below, 40-8-32 DU is the formulation now proposed for routine use.

In a trial performed in 1982-1983, 96.6 percent of infants had detectable circulating antibody to all three poliovirus types one month after a single dose at two months of age (table 1). These initial titers could represent either response to vaccination or residual maternal antibody. A second dose was given at three-and-a-half to four months of age: within one week all children had high antibody titers for all three poliovirus types. Geometric mean antibody titers rose slightly from one week to one month after the second dose. The rapidity and magnitude of the antibody response following the second dose is consistent with a secondary-type response following immunologic priming by the first dose. (As shown in figure 4, the magnitude of a secondary response is relatively small early after priming, compared with later.) Immunologic memory was tested again six months later by administering a challenge dose of killed poliovirus vaccine at nine-and-a-half to ten months of age: all children rapidly responded with very high titers.

FORMULATION OF POLIOVIRUS VACCINE FOR ONE-DOSE EFFECTIVENESS

We have evaluated trivalent poliovirus vaccines, prepared from the RIVM monovalent pools, that ranged in antigen content from 5 to 320 DU, 0.5 to 32 DU, and 1 to 64 DU of poliovirus

types 1, 2, and 3, respectively. To induce immunity to paralysis reliably with a single dose, we propose poliovirus vaccine formulated to contain 40 DU of type 1, 8 DU of type 2, and 32 DU of type 3 poliovirus.

When administered to six month old infants, a single dose of such vaccine induces circulating antibody titers of 1:4 or greater in 95 percent, 100 percent, and 90 percent of subjects against poliovirus types 1, 2, and 3, respectively (fig. 6). Serum antibody titers reach a maximum two weeks following immunization, after which they persist at the same level or decline to a lower plateau (10): six months after a single dose of 40-8-32 DU poliovirus vaccine, antibody titers ≥1:4 against types 1, 2, and 3 poliovirus are present in 95 percent, 95 percent, and 51 percent of subjects, respectively (6). Despite the decline in geometric mean antibody titers against type 3, immunologic memory has been induced in all children: one month after challenge with vaccine all subjects have antibody titers of 1:32 or greater for all three poliovirus types (fig. 6).

Immunologic memory can be demonstrated in essentially all children one to two months after a single dose of potent vaccine at six months of age (fig. 4). When administered at two months of age, a single dose of 40-8-32 DU vaccine may also induce immunologic memory in all infants (table 1): certainly one dose at two months and one dose at three-and-a-half to four months induces both circulating antibody and immunologic memory in all.

The presence of immunologic memory, with or without

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detectable circulating antibody, provides long-term protection against paralytic poliomyelitis (10). A single dose of killed poliovirus vaccine containing antigen equivalent to the 40-8-32 DU reference vaccine can therefore be expected to induce lifelong immunity when administered after six months of age. Available data also suggest that one dose of 40-8-32 DU vaccine may be effective in younger infants; the RIVM reference vaccines are now being titrated in newborns to determine unequivocally the antigen content needed to ensure one-dose immunization early in life.

DISCUSSION

Using modern methods of vaccine production and standardization (3, 11, 12), the antigen content of killed poliovirus vaccine can be adjusted to achieve any desired response. In this paper, we describe the performance of a vaccine that may be used as a reference standard against which killed poliovirus vaccines can be measured. This reference vaccine could also be used to standardize noninfectious vaccines produced by technologies now under development: splitting viruses into subunits, chemical synthesis of viral peptides, and production of viral protein by recombinant DNA techniques.

Killed poliovirus vaccine distributed in the United States between 1955 and 1959 was approximately 55 percent effective in protecting against paralysis following a single dose. Killed vaccine produced both before and after this period was of greater potency (1, 2, 13-15). Vaccine that is 100 percent effective with a single dose can now be formulated reliably (fig. 7).

Killed poliovirus vaccine available for many years has been fully effective following two or three doses. This schedule can be conveniently used in developed countries with established programs of immunization because poliovirus vaccine is administered in parallel with DTP vaccine, either as a separate vaccine or in a combined DTP-polio vaccine. The availability of high potency killed poliovirus vaccine allows for a choice of immunization program. Killed vaccine could be administered by itself in a single dose at a convenient age, or it could be used in a two- or three-dose schedule combined with DTP, if this is indicated for practical reasons in a specific setting.

The reference poliovirus vaccine we describe here has already been used to standardize a commercially produced 40-8-32 DU poliovirus vaccine (12, 16). This commercial vaccine is licensed and used in a multidose schedule in combination with DTP vaccine in France, The Netherlands, and elsewhere; application for license has been made in the United States.

Flexibility of schedule and reliable protection after the first dose are special advantages in developing countries where reduction of the number of doses required for full immunization leads to more efficient use of limited health care resources. Even though it is presently more expensive than oral live poliovirus vaccine, use of killed poliovirus vaccine can be expected to reduce the cost and increase the coverage achieved by immunization programs in developing countries because fewer doses are needed for effective immunization (17).

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Combined DTP-polio vaccines are now being used in routine vaccination programs in Senegal (18) and Upper Volta, where the first of two doses is administered by mobile teams at three to eight months of age, and the second is administered approximately six months later. Good coverage has been achieved with these simple, routine programs. In Upper Volta, the World Health Organization recently sponsored an evaluation of the program. In two rural areas, after only two visits by a vaccination team, 77 percent and 63 percent of children had received one and two doses of vaccine, respectively. In an urban area, after three team visits, 92 percent and 80 percent of children had received one and two doses, respectively. The diphtheria, tetanus, and even the pertussis components are gratifyingly effective in such a two-dose schedule (8, 19).

Transmission of poliovirus is reduced with use of killed poliovirus vaccine: a herd effect has been demonstrated in many field studies in the United States and Europe even when as little as 15 percent of a population has been immunized (20-23). Use of killed vaccine has eradicated disease and eliminated circulating polioviruses from large geographical areas in the developed world (20, 24). Although it has not yet been applied as widely in any developing country, similar control of poliovirus transmission can be expected because administration of potent killed poliovirus vaccine reduces both fecal and pharyngeal transmission of poliovirus (20, 23-26).

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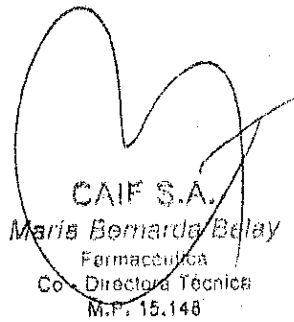
Table 1. Geometric mean antibody titers among infants who received killed poliovirus vaccine in Israel in 1982-1983. One month after the first vaccination, 96.6 percent of subjects had antibody titers ≥1:2 for each poliovirus type; at all other times, 100 percent of subjects had detectable antibody. The vaccine contained 40, 8, and 32 D-antigen units of poliovirus types 1, 2, and 3, respectively. Vaccine was administered at 2, at 3-1/2 to 4, and at 9-1/2 to 10 months of age.

	First Vaccination	Second Vaccination		Third Vaccination
Time after vaccination:	1 month	1 week	1 month	1 week
No. subjects:	29	36	76	72
Poliovirus Type 1	6	152	205	1139
Poliovirus Type 2	8	103	145	979
Poliovirus Type 3	9	465	751	1388



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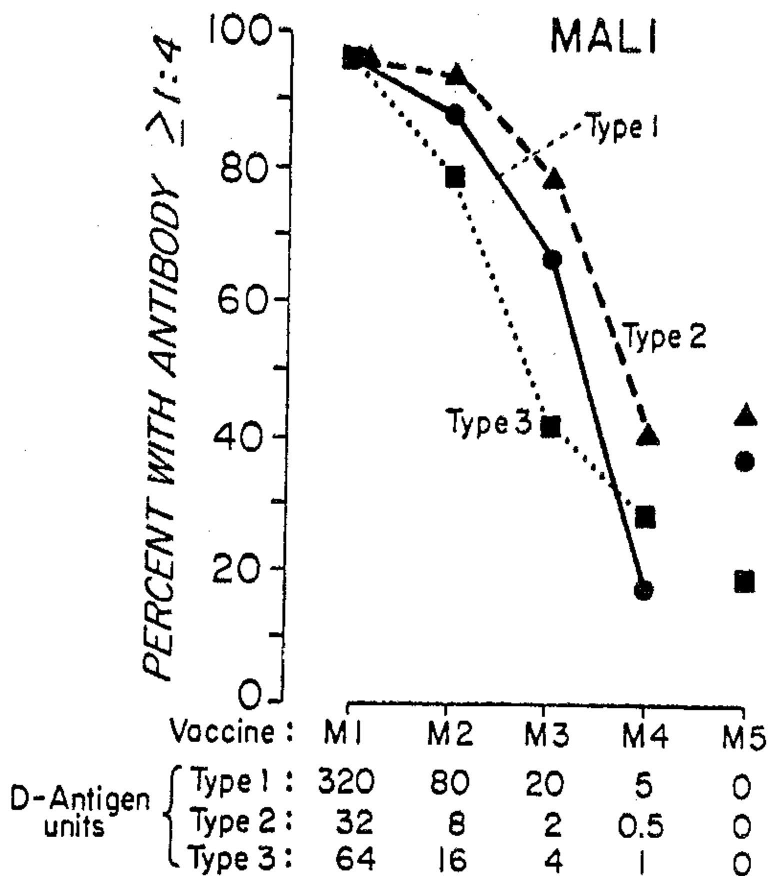

Figure 1 Percentage of children in Mali (3-10 months old) with antibody titers ≥1:4 one month after receiving one dose of killed poliovirus vaccines containing different quantities of antigen. Groups M1-M5 contained 26, 34, 33, 29, and 28 subjects, respectively. (M5: tetanus toxoid.) (Data from Salk et al. [4]: results for nonadsorbed vaccine.)

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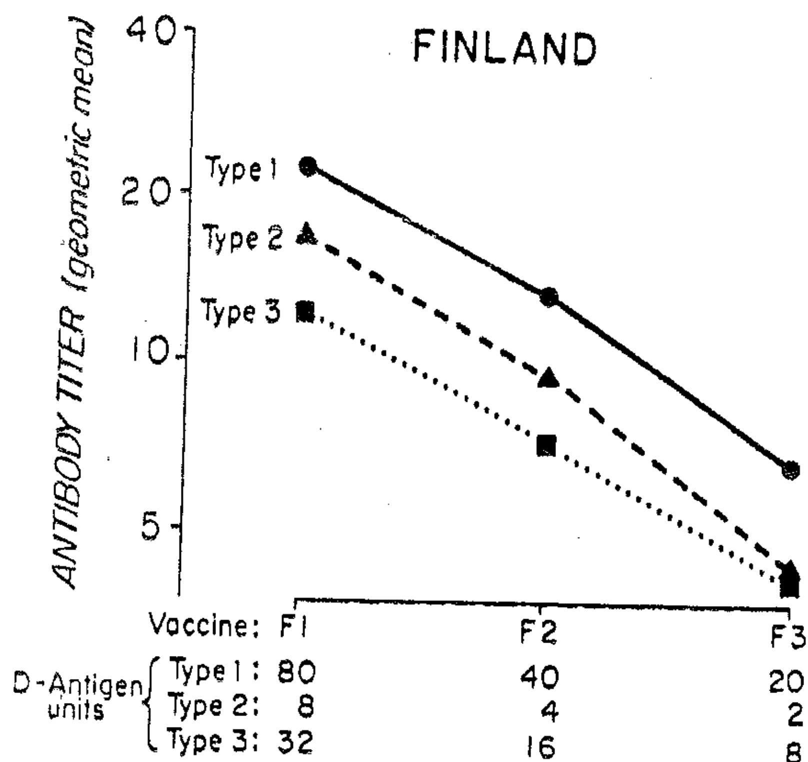

Figure 2 Geometric mean antibody titers (reciprocal of serum dilution) in Finland one month after one dose of killed poliovirus vaccines containing different quantities of antigen. Approximately 100 subjects six months old were in each of the three vaccine groups. (Adapted from Salk et al. [5], with permission.)

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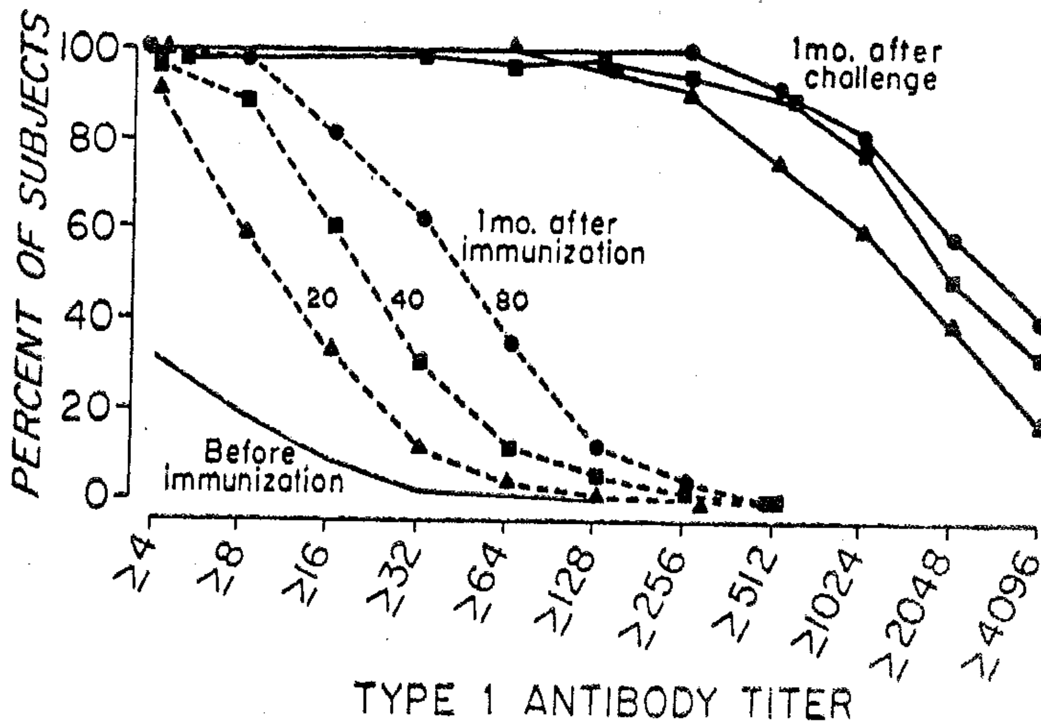

Figure 3 Type 1 antibody titers (reciprocal of serum dilution) in infants in Finland immunized with one dose of killed poliovirus vaccine at six months of age and challenged with vaccine six months later. Residual maternal antibody present before immunization. (Number of subjects for 20, 40, and 80 DU, respectively: 38, 103, 39.) (Adapted from Salk et al. [6], with permission.)

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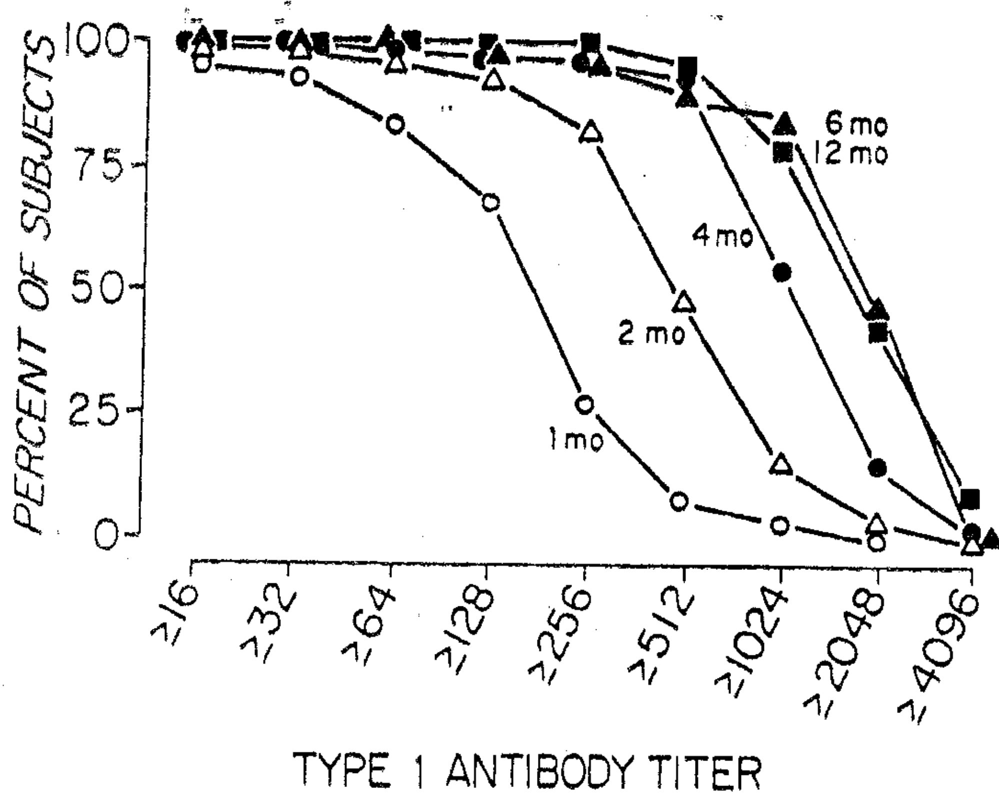

Figure 4 Type 1 antibody titers (reciprocal of serum dilution) in infants in Finland one month after a single challenge dose of vaccine administered one to twelve months after immunization at six months of age with a single dose containing 80 DU type 1. (Number of subjects in 1-, 2-, 4-, 6-, and 12-month challenge groups, respectively: 71, 98, 103, 91, 87.)

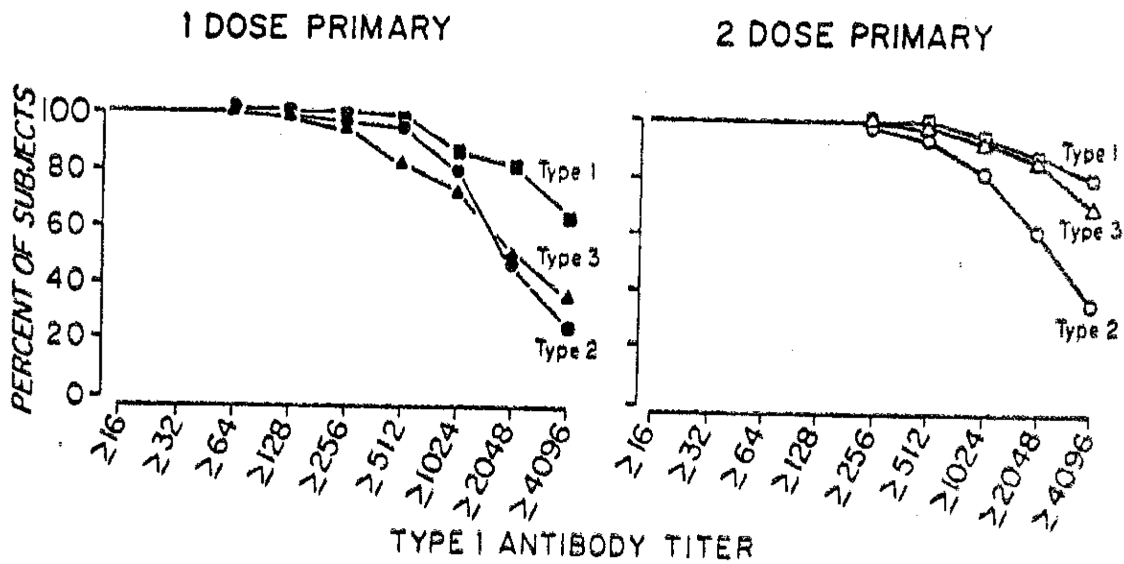

Figure 5 Antibody response (reciprocal of serum dilution) of infants in Sweden to challenge with vaccine six months after primary immunization at seven to nine months of age with either one or two doses of 40-8-32 DU killed poliovirus vaccine. (Number of subjects for types 1, 2, and 3, respectively: one dose primary: 68, 54, 54; two dose primary: 15, 49, 50.)

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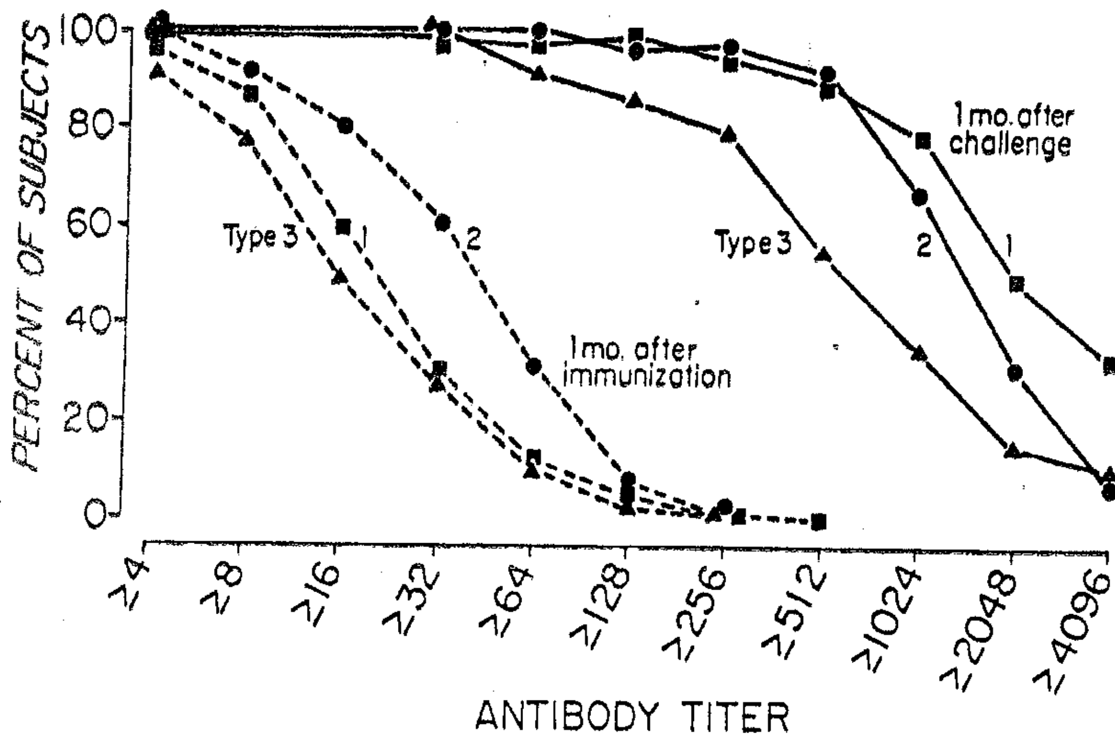

Figure 6 Distribution of antibody titers (reciprocal of serum dilution) after one immunizing dose and one six-month challenge dose containing 40, 8, and 32 D-antigen units of types 1, 2, and 3, respectively. (Number of subjects: 109, 39, and 39, respectively.) (Adapted from Salk et al. [6], with permission.)

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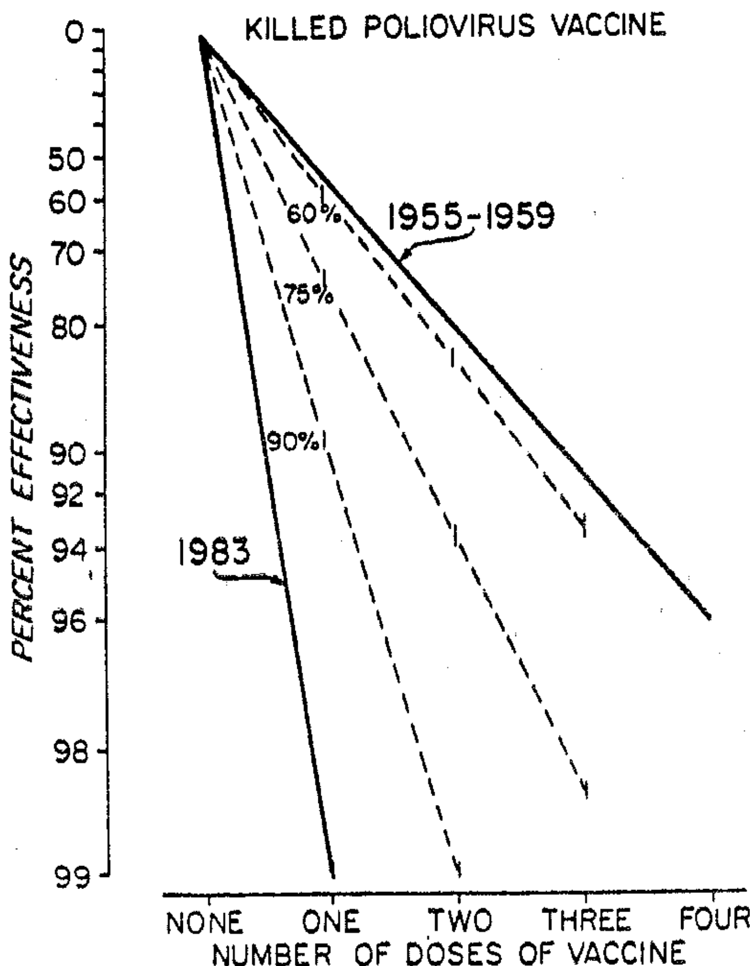

Figure 7 Killed poliovirus vaccine used in the United States between 1955 and 1959 was approximately 55 percent effective in preventing paralysis following a single dose (1). A 40-8-32 DU killed poliovirus vaccine is expected to approach 100 percent protection with a single dose. (Adapted from Salk [1], with permission.)

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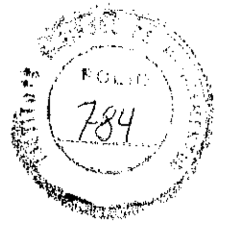

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IMMUNOLOGIC MEMORY INDUCED AT BIRTH BY IMMUNIZATION WITH INACTIVATED POLIO VACCINE IN A REDUCED SCHEDULE

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Key words: IPV immunization - Immunization at birth - Immunological memory

One hundred forty-one healthy newborns were immunized 24 hours after birth with one dose of inactivated polio vaccine (IPV) of enhanced potency. Following the administration of a second vaccine dose six months later, a considerable proportion of babies responded with neutralizing antibody (NA) to the three poliovirus types. The very rapid occurrence and high antibody titer were indicative of an anamnestic response. Twenty-one infants who still had NA < 1:4 to one-more poliovirus types after the second vaccine dose responded with very high NA values 7-10 days after a supplementary dose of IPV. It appears that IPV of enhanced potency administered at birth is apt to induce immunologic memory, which should provide the basis for protection against paralytic poliomyelitis in case of exposure to wild poliovirus later in life.

INTRODUCTION

Paralytic poliomyelitis in the first year of life is still a disease of importance to the developing countries (3, 5), where the conventional use of oral polio vaccine (OPV) has brought about only a partial answer (1, 2, 4). Successful control of paralytic poliomyelitis in the developing countries depends on the use of a potent antigen that requires few doses in very young children, and which is capable of inducing immunologic memory. We report results obtained from the administration of one dose of a highly potent inactivated polio vaccine (IPV) at birth followed by a second dose given in the second half of the first year of life.

MATERIALS AND METHODS

Three groups of healthy newborns (A, B and C) from an area where wild poliovirus type 1 is present were immunized 24 hours after birth with one of three IPV[2] of different antigenic potency: 160-32-128, 80-16-64 and 40-8-32 D-antigen units for poliovirus types 1, 2

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TABLE 1. - Immune Status in Neonates Immunized with I.P.V. - Israel, 1985-1986

Group	On the day of the first injection (Age: 24 H)			3½ months after the first injection (Age: 3½ mo)			At day of booster (Age: ≧ 6 mo)			One week after booster			One month after booster		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
A: 48 neonates															
% seropositives*	100.	100.	100.	64.3	80.9	85.7	20.0	30.0	42.5	89.2	86.5	94.6	81.3	89.6	77.1
Geometric mean	121.8	199.5	90.5	3.6	8.6	16.0	2.1	2.7	3.9	108.1	75.8	416.7	47.9	58.7	65.9
B: 50 neonates															
% seropositives	100.	100.	100.	78.7	76.6	74.5	29.8	34.0	38.3	85.7	97.1	91.4	78.0	100.	90.0
Geometric mean	152.9	266.3	95.1	5.7	7.0	10.3	2.6	3.2	3.4	35.3	84.5	130.6	29.4	67.4	71.5
C: 49 neonates															
% seropositives	100.	100.	100.	66.6	75.5	66.6	29.5	27.3	20.5	85.4	79.4	94.1	79.6	97.9	71.4
Geometric mean	98.1	194.1	107.6	4.2	8.6	5.4	2.5	3.4	1.9	78.5	42.6	66.4	44.3	106.5	24.5

* Seropositive: Polio neutralizing antibody titer ≧ 1:4

and 3, respectively. After the age of 6 months, the infants were given a uniform dose of vaccine with 40-8-32 D-antigen units for each of the three poliovirus types, respectively. Neutralizing antibody (NA) was measured in the umbilical cord blood and in capillary blood at the age of 3 ½ months, on the day of the second dose, and one week and one month later by neutralization assay in microtitration plates (7).

RESULTS

The data in Table 1 show a decline in the proportion of children with NA ≧ 1:4 and in the geometric mean values (GMV) from birth to the age of six months, at which time only one third or fewer of the infants still had circulating antibodies. One week after the second dose of vaccine the percent of seroresponders and the increase in the GMV were considerable. One month after the second dose of vaccine there was a small decrease in the proportion of infants with demonstrable antibodies to types 1 and 3, and there was a decrease in the GMV to all three types, although still much above the level needed for protection. There was no apparent difference in the immune response following the use of antigens of different potency.

All 21 infants with NA < 1:4 to one or more antigenic type after the second dose of vaccine were given a supplementary dose of IPV with 40-8-32 D-antigen units. As shown in Table 2, seven to ten days later an antibody response was present in all children, mostly with high titers, characteristic of an anamnestic response.

TABLE 2. - Immune Status of 21 Infants with Polio Neutralizing Antibody < 1:4 to One or More Poliovirus Types, Given a Second IPV* Booster

	Before the second booster			7-10 days after the second booster		
	1	2	3	1	2	3
Percent of seropositive	23.6	66.1	52.4	100.	100.	100.
Geometric mean level	2.7	13.1	10.1	958.6	1854.9	2580.3

* 40-8-32 D ag. units

DISCUSSION

The high proportion of infants responding with high antibody titers one week after administration of a second dose of IPV, as well as the rapid, high level response after a supplementary dose of vaccine, point to the presence of immunologic memory that had been induced at birth with one dose of potent killed poliovirus vaccine (6). Such a rapid and high response would provide protection against paralytic disease in case of exposure to a wild virus later in life. Thus, a single dose of potent IPV administered at birth could reduce the incidence of paralytic poliomyelitis in developing countries, even if subsequent doses of poliovirus vaccine fail to be administered because of economic, social, or other practical considerations.

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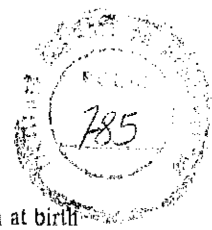

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IMMUNE RESPONSE TO INACTIVATED POLIO VACCINE ADMINISTERED IN A TWO DOSE SCHEDULE

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Introduction

Developments in the technology of inactivated poliovirus vaccine (IPV) production which have made it possible to increase its efficacy (1) have led to the use of a modified vaccine schedule consisting of a primary immunization based on the administration of two successive doses early infancy, followed by a reinforcing dose given 6-12 months later (2,3). On this occasion, it was repeatedly observed that after the second IPV dose, in a high proportion of children an immune response of anamnestic type was present. Further studies have shown that infants whose age ranged between 2-24 months, given one dose of IPV of enhanced potency, responded in a high proportion with production of neutralizing antibody to a second dose of IPV (4-8).

Since in several areas of the world, infants are still vulnerable to paralytic poliomyelitis, mainly because of logistic obstacles to completing the vaccination schedule, and because of immunization failures following the use of oral polio vaccine (OPV), it was considered worthwhile to evaluate the immune response to a simplified IPV schedule by using a potent polio antigen which, administered in early infancy, would sensitize the immune system for an anamnestic response to a second antigenic dose given six months later.

Material and Methods

Two groups of 61 and 68 healthy infants were primed at the age of 2 months with one dose of either 160-32-128 or 80-16-64 D antigen units, corresponding to the three poliovirus types, respectively. Six months later both the groups were boostered with one dose of the standard 40-8-32 D ag units. As a control group, 79 infants were administered two doses of 40-8-32

D ag u at the age of 2 and 3½ months and boostered at 9 months, which is the routine polio immunization schedule in a limited area of Israel.

The vaccine in three different concentrations of D antigen units: 160-32-124, 80-16-64 and 40-8-32 for each of the three polio virus types, respectively was prepared at the Dutch National Institute of Public Health at Bilthoven.

Bloods were collected on the day of the first injection, one month later, at the date of the booster, and one week, one month and one year after it.

Polio neutralizing antibody (NA) were measured by neutralization assay in microtitration plates - microneutralization test. Antibody were considered present when a titer of ≥ 1:4 was found.

As shown in Table 1, a considerable proportion of infants in the study and control groups carried low titers of maternal antibody.

Results

One month after priming, NA were present in a considerably higher percent of infants given 160-32-124 and 80-16-64 D ag.u. vaccine, respectively, than in the control group immunized with the standard 40-8-32 D ag.u. vaccine (see Fig. 1). In contrast, geometric mean (GM) values were uniformly low in the three groups.

At the date of the second vaccine dose administration, the percentage distribution of NA ≥ 1:4 and the GM values in children primed with one dose of 160-32-124 and 80-16-64 D ag.u. were clearly lower that at the date of start of immunization.

One month after second dose of vaccine, an very high antibody response, in parallel with considerable GM values, was observed in the three groups.

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A dose effect (highest values in the control group, lowest values in the study group given 80-16-64 D ag.u. vaccine) was noted, particularly in response to type 1 and type 2, and less for type 3.

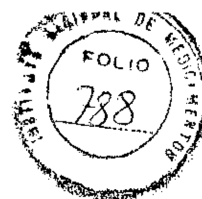
Follow-up to one year after the second dose of vaccine revealed practically no change in the percent distribution of NA ≥ 1:4. Simultaneously, there was a considerable drop in GM levels, particularly for types 1 and 3, in the study group, as compared with a more attenuated decline observed in the control group. Nevertheless, GM levels of protective value against the three types of polio virus were found.

The booster response to the administration of an antigen of lower potency was demonstrable, as seen in Figure 2, by the cumulative percent distribution of polio NA antibody to type 1: one week after the reinforcing dose, the considerable proportion of high titers of NA antibody observed was very similar to the percent distribution of antibody observed one month after the booster dose.

A similar pattern was observed for NA to type 3 poliovirus, as shown in Figure 3, which indicated the occurrence of an intense and rapid antibody response of anamnestic type.

Comment

The very high proportion of babies in the study group with NA ≥ 1:4 one month after the first vaccine dose administration, in spite of the high percentage of infants covered by maternal antibody at the date of priming, demonstrates again that a primary immune effect can be accomplished when immunization is started at the age of 2 months.



The evident decrease in the percentage distribution of polio NA to the three poliovirus types six months after priming, observed elsewhere also (2,3) supports the probability of absence of exposure of the study group to natural infection up to the date of booster.

After the second dose of antigen, high titers of NA antibody to the three types of poliovirus were present in 100% of immunized babies, as reported in several previous trials with IPV of enhanced potency (3-8) administered in a two dose schedule or following the second dose if more than two IPV doses were used. In most of these studies, in which the standard 40-8-32 D ag.u. vaccine was mostly used for both priming and booster, the age at first vaccine administration as well as the interval until the booster date varied considerably, as shown in Table 2.

In the present trial, antigens of higher than usual potency were used for priming with the purpose of stimulating the immune system with an amount of antigen adequate to induce an immunizing effect, as it is agreed that the immunological memory and the degree of humoral antibody response to a booster are determined, among others, by the antigenic content of the vaccine employed (9,10) in the primary immunization. There is a dose response relationship between the quantity of antigen used in the primary stimulation and the level of both primary and secondary antibody response (10). A single dose of IPV with a sufficiently high quantity of antigen was capable of uniformly sensitizing the immune system to respond adequately and consequently the 40-8-32 D ag.u. IPV concentration in the booster dose was enough to induce an immune response in 100% of the infants. On the other hand, by comparing the percentage distribution of NA and the GM values after priming with 160-32-128 and 80-16-64 D ag.u. vaccine, it was evident that over the critical range of 80-16-64 D ag.u. concentration, there is a linear

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relationship between the antibody response and the quantity of antigen administered, as stated elsewhere (10). In this respect, one should mention that a 40-8-32 D ag.u. concentration in a two dose schedule proved to be effective in producing a satisfactory antibody response (5,8).

Similarity of humoral antibody levels at one month and one week after booster indicates the presence of immunological memory, revealed by the rapid and high level of antibody response of anamnestic type.

Since larger doses of antigen induce a degree of immunological memory equivalent to that following a homotypic infection, the high content of the three polio antigen types in the vaccine used for priming is also supposed to produce a solid immunological memory to each of the three homotypic infections (11). As the degree of immunological memory varies with the size of the first dose of IPV (10,12), it appears that the use in this study of polio antigens of enhanced potency would contribute to a persistent immunological memory and ensure a durable immunity.

It could be questioned as to whether presence of protective values of polio NA in all the children in the study group one year after booster constitutes definite proof of a persistent immunological memory. On the other hand, the field monitoring of routine IPV programs has indicated long term persistence of seroimmunity (13) and recent personal observations show that following immunization in infancy with a 40-8-32 D ag.u. vaccine in two basic doses and a booster, a protective level of NA antibody was maintained in all children up to 7 years after completion of immunization.

As protection against paralytic disease is associated with presence of either type specific serum antibody or type specific immunological memory, which once induced is irreversible (12), the anamnestic response following the second dose of vaccine observed in this study may be an indicator of

what would occur after a challenge with natural infection (11). It is very probable that on exposure to infection later in life the immunological memory would recall sufficiently quickly in a case where the humoral antibody level has declined below the protective level.

The results of this study indicate that a two dose immunization schedule based on priming with a potent antigen at 2 months of life followed by a booster six months later is a simple and advantageous formula: it is protective after the first dose administered early in life, before effective immunity can be induced by immunization regimens with oral polio vaccine requiring repeated doses of antigen; it requires a lowest number of immunizing doses; it allows the association of polio antigen with other inactivated vaccines. Furthermore, with a 100% effectivity in terms of induced immunological memory produced after the first dose of vaccine, the program could be especially useful in endemic areas.

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INACTIVATED POLIO VACCINE STUDY DESIGN
ISRAEL 1985-1986

Group	Number of children	Antigenic content* of vaccine at date of immunization		
		2 mo.	3½ mo.	9 mo.
Study				
A	61	160-32-128	—	40-8-32
B	68	80-16-64	—	40-8-32
Control	79	40-8-32	40-8-32	40-8-32

* D antigen units for types 1, 2 and 3 respectively

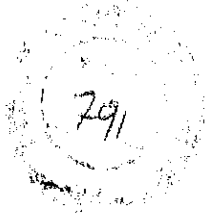

Table 1

POLIO IMMUNE STATUS OF INFANTS AT THE DATE
OF FIRST IPV ADMINISTRATION*

Group	Percent of infants with antibody** to type			Geometric mean titer		
	1	2	3	1	2	3
Study						
A	71.4	85.7	38.1	4.3	13.2	4.7
B	86.6	87.1	60.0	7.3	10.1	4.4
Control	66.7	80.0	50.0	3.9	10.1	1.9

* Age of 2 months

** Neutralizing antibody ≥ 1.4

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Figure 1

Percentage Distribution of Polio Neutralizing Ant
in Three Immunization Sch

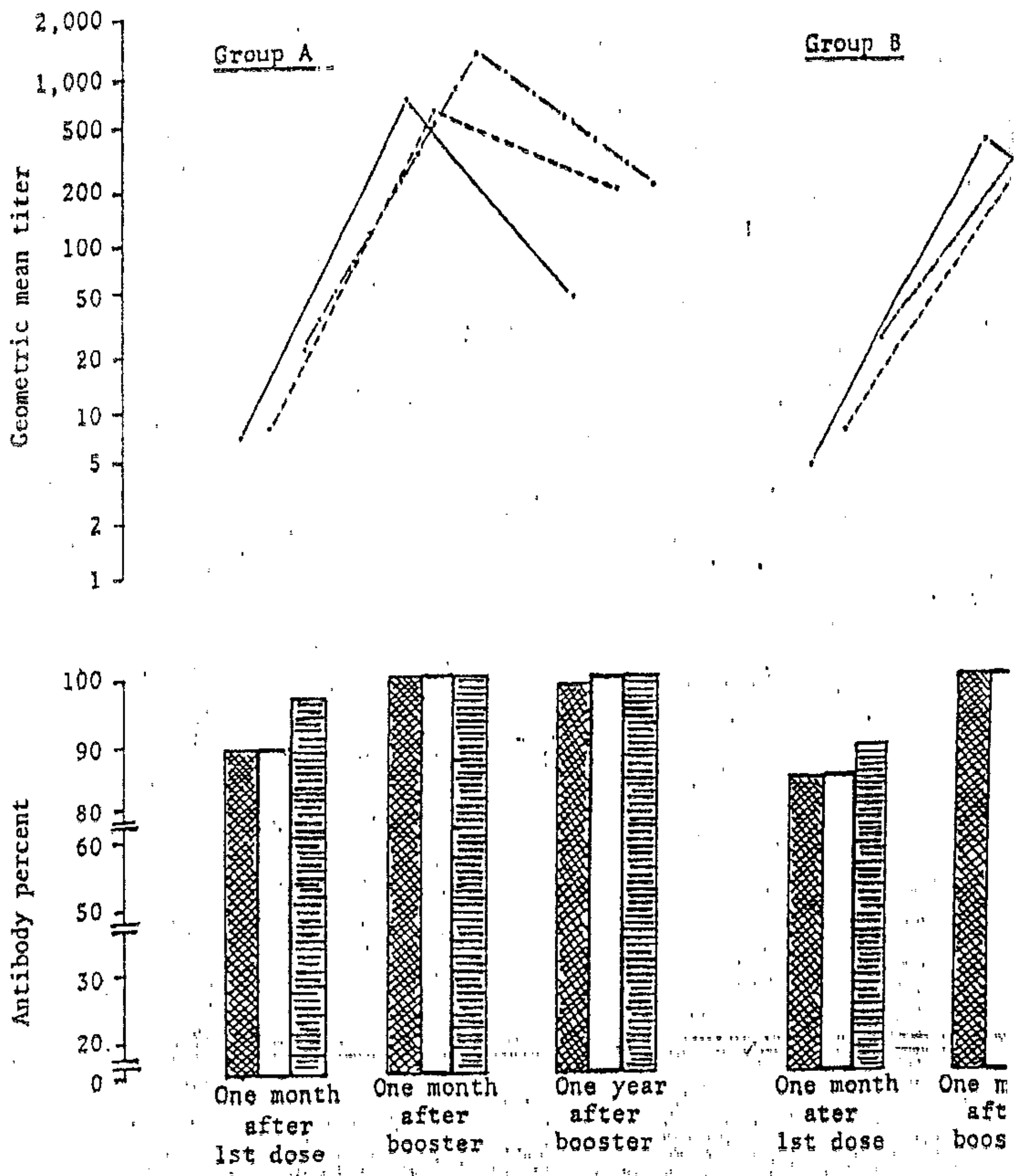

Figure 1

lio Neutralizing Antibody (≥ 1:4) and Geometric Mean Titer
ree Immunization Schedules with I.P.V.

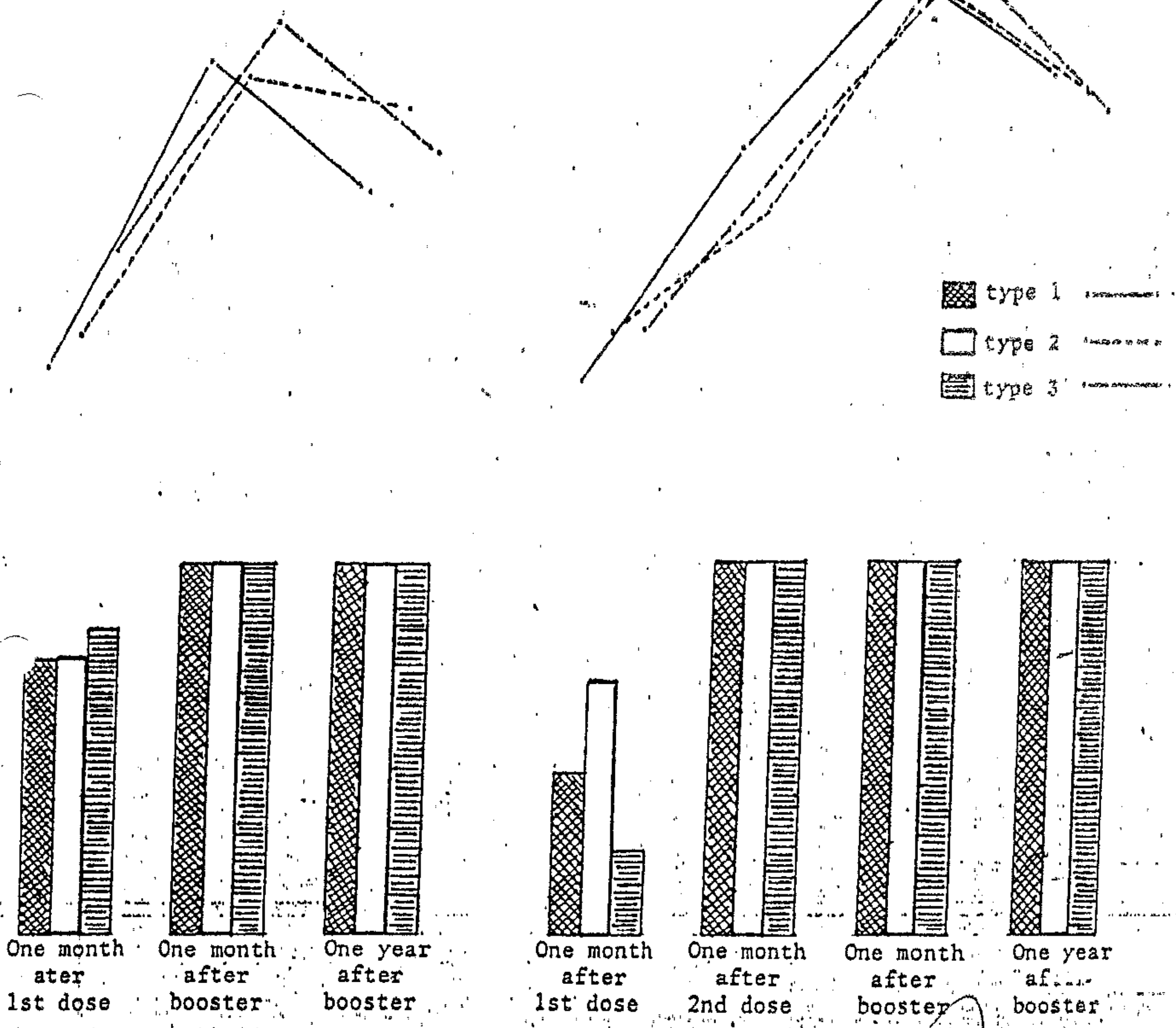

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Figure 2

Cumulative Percentage Distribution of Type 1 Polio Neutralizing Antibody following Administration of IPV

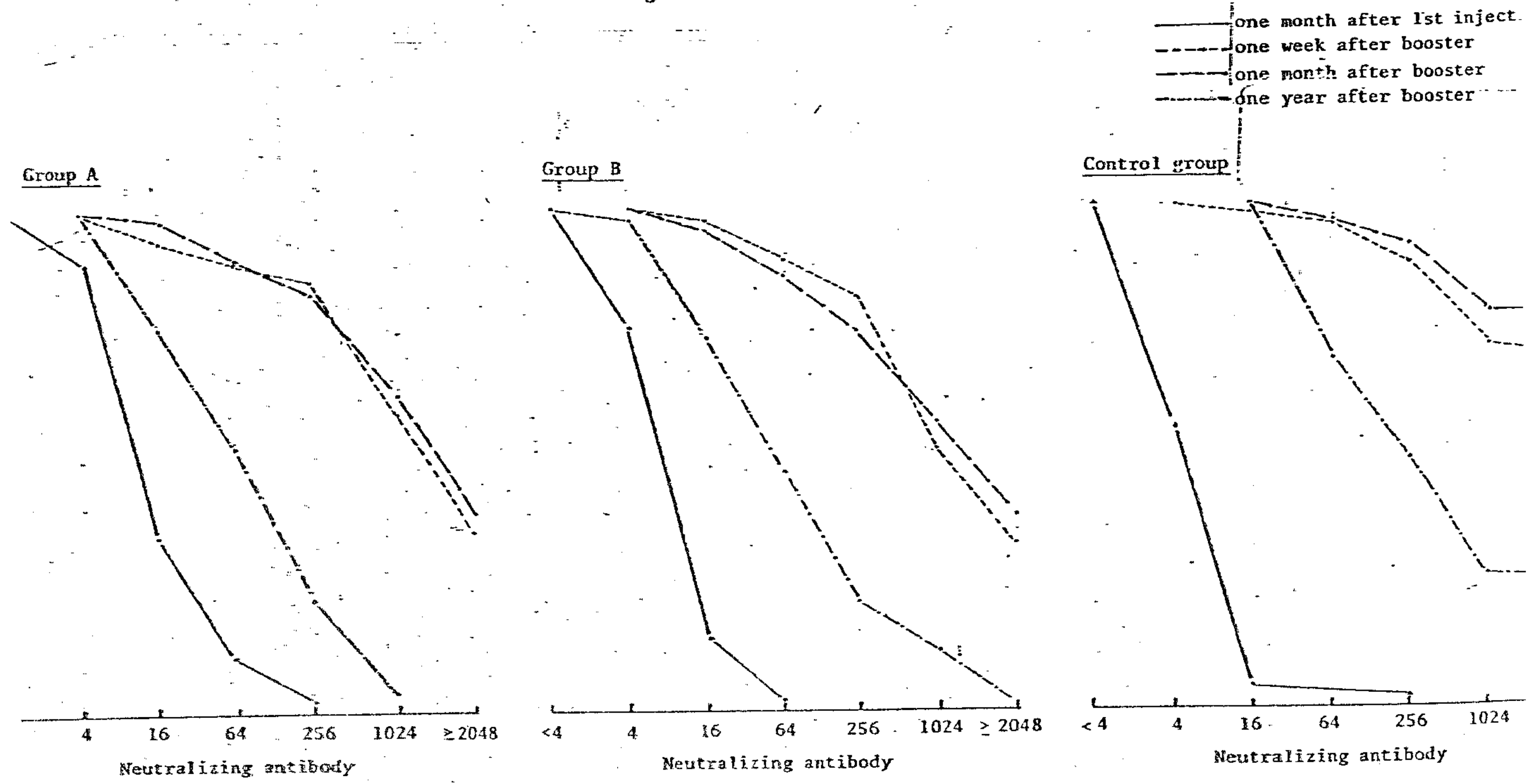

Figure 3

Cumulative Percentage Distribution of Type 3 Polio Neutralizing Antibody following Administration of IPV

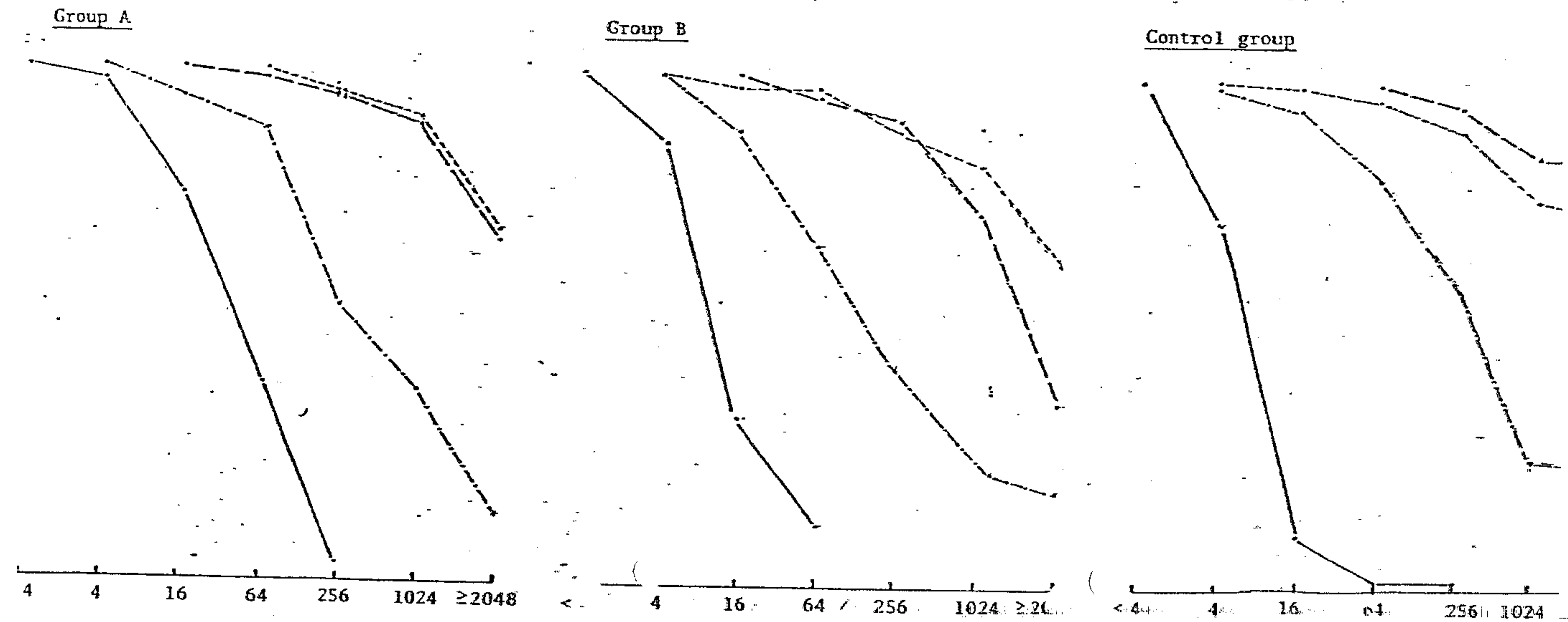

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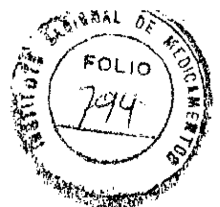
Table 14

Immune Response in Three Groups of Infants Immunized with I.P.V.

	One month after 1st injection (age at administration: 2 mo.)			One month after 2nd injection (age at administration: 3½ mo.)			Six months after priming (age: 8 mo.)			Booster (age at administration: 8 mo.) one week after			one month after			one year after		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
A: 65 infants primed with one dose of 80-16-64 and boosted with 40-8-32 D Ag. u.																		
– N.A.*	85.0	87.5	90.0	—	—	—	58.1	67.4	67.4	100.	100.	100.	100.	100.	100.	90.8	95.1	93.8
– G.M.**	3.7	6.7	20.9	—	—	—	5.5	8.1	5.5	461.4	404.1	1024.0	467.9	321.2	707.0	20.1	78.0	40.7
B: 61 infants primed with one dose of 160-32-128 and boosted with 40-8-32 D. Ag. u.																		
– N.A.	89.4	89.4	97.4	—	—	—	83.0	80.5	80.5	100.	100.	100.	100.	100.	100.	90.4	97.4	100.
– G.M.	6.8	7.4	20.9	—	—	—	12.6	11.3	10.3	541.6	308.7	1381.9	653.5	424.6	1426.5	23.9	165.2	153.
C: 79 infants primed twice and boosted with 40-8-32 D. Ag. u.																		
– N.A.	51.0	80.0	27.0	100.	100.	100.	96.7	95.0	96.7	100.	100.	100.	100.	100.	100.	100.	100.	100.
– G.M.	3.9	10.1	11.9	141.6	65.6	173.5	32.6	19.1	16.9	1118.1	992.2	1443.0	1383.0	1300.7	1582.9	478.4	215.3	144.

* Percent of infants with N.A. ≥ 1:4

** Geometric mean titer



Next Generation Inactivated Polio Vaccine Manufacturing to Support Post Polio-Eradication Biosafety Goals

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Abstract

Worldwide efforts to eradicate polio caused a tipping point in polio vaccination strategies. A switch from the oral polio vaccine, which can cause circulating and virulent vaccine derived polioviruses, to inactivated polio vaccines (IPV) is scheduled. Moreover, a manufacturing process, using attenuated virus strains instead of wild-type polioviruses, is demanded to enhance worldwide production of IPV, especially in low- and middle income countries. Therefore, development of an IPV from attenuated (Sabin) poliovirus strains (sIPV) was pursued. Starting from the current IPV production process based on wild type Salk strains, adaptations, such as lower virus cultivation temperature, were implemented. sIPV was produced at industrial scale followed by formulation of both plain and aluminium adjuvanted sIPV. The final products met the quality criteria, were immunogenic in rats, showed no toxicity in rabbits and could be released for testing in the clinic. Concluding, sIPV was developed to manufacturing scale. The technology can be transferred worldwide to support post polio-eradication biosafety goals.

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Introduction

Vaccines that provide protection against poliomyelitis have been available for decades [1]. Yet large efforts are undertaken in WHO's global polio eradication initiative (GPEI) to obtain the next generation vaccines that are safe and available at low costs [2]. These vaccines are needed both for the 'endgame' in polio eradication and after eradication to prevent the risk of a global outbreak due to accidental or deliberate re-introduction of the virus. One of the anticipated next generation vaccines is an inactivated polio vaccine (IPV) based on the attenuated Sabin poliovirus strains resulting in a so-called Sabin-IPV (sIPV) [3]. The Sabin polioviruses (PV) are currently used in live oral polio vaccines (OPV) [4] and will provide additional bio-safety, over the wild-type viruses, during the manufacturing process [5]. Bio-safety requirements are becoming more stringent as new containment guidelines are drafted by the WHO's Global Action Plan for Wild Poliovirus Laboratory Containment III (GAPIII) [6]. Future production and quality control of IPV using wild-type strains will require at least biosafety level 3 facilities [7]. This will not only increase manufacturing costs but will also limit the possibility of IPV manufacturing in low- and middle income countries for instance due to requirements on immunization coverage. The use of alternative strains like Sabin PV would require less stringent biocontainment, is encouraged by the WHO [5] and allows manufacturing in low- and middle income countries, which potentially lowers manufacturing costs [8]. Moreover, the use of an IPV instead of OPV will prevent the emergence of circulating vaccine-derived PV (cVDPVs), which may potentially re-seed the world with PV and negate the GPEI accomplishments [9].

The development of the currently used IPV production process (for a process overview see Figure 1) dates back to the 1960s when at the RIV in Bilthoven a process was developed based on micro-carrier technology and primary monkey kidney cells [10,11]. This process was scaled-up to 350-L and later 750-L bioreactors. Additionally, the Vero cell line was introduced to replace the then used tertiary monkey kidney cells. To support manufacturing and increase the knowledge on IPV manufacturing, efforts like multivariate data analysis and the development of scale-down models, i.e. lab-scale equivalents of the manufacturing-scale processes, have been undertaken [12,13]. The availability of scale-down

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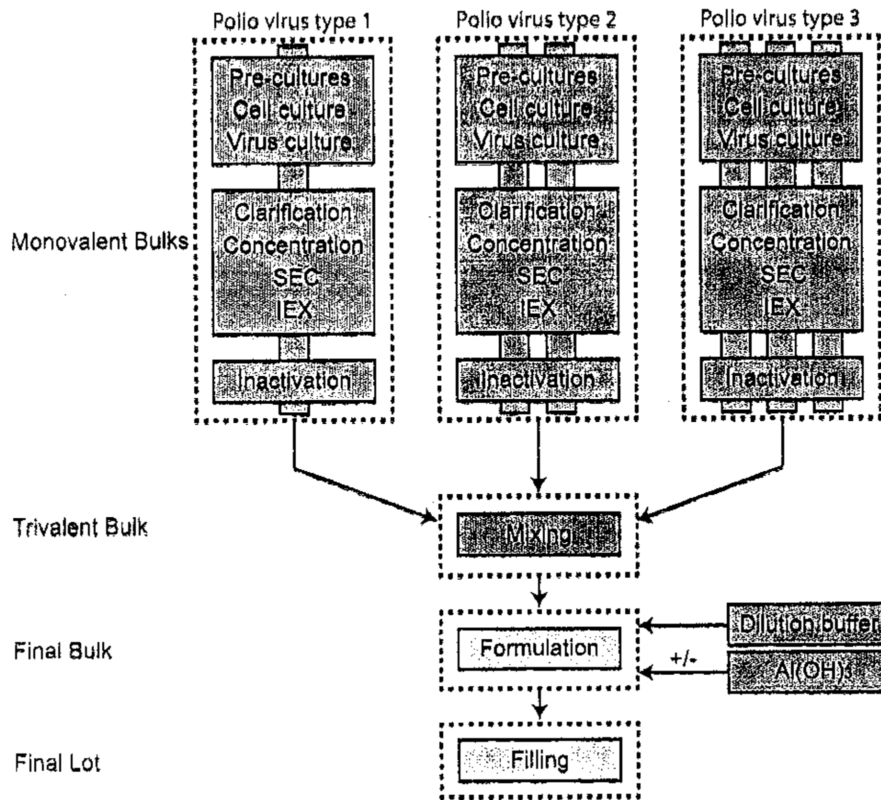

Figure 1. **Process overview for preparation of trivalent IPV.** Monovalent bulks are prepared for each PV (type 1, 2 and 3) separately. During monovalent bulk preparation Vero cells are expanded using two pre-culture steps and a cell culture followed by virus culture. Virus is purified using normal flow filtration for clarification, tangential flow filtration for concentration and two chromatography units, size exclusion and ion exchange chromatography. Purified virus is subsequently inactivated using formaldehyde. Subsequently these are mixed to obtain trivalent bulk prior to formulation and filling.
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models, unique in the vaccine world, allows rapid assessment of process changes.

Based on our vast history in IPV development and production [11], our previous experience with sIPV [14] and technology transfer [15-17] a project for the development and technology transfer of sIPV manufacturing under supervision of the WHO was initiated. Initially a proof-of-principle study was performed. In this study, sIPV was prepared from OPV as virus source. The three PV sub-types, obtained separately (Bio Farma, Indonesia), were concentrated, purified and inactivated and shown to yield a sIPV that was immunogenic in an animal model [3]. In principle, OPV manufacturers could, by acquiring correct downstream processing (DSP) equipment, produce sIPV. However, larger quantities of virus harvest (100-800 fold of current production quantities) are needed and upstream processing (USP) should be scaled-up [18].

Here we report the results of limited (to be able to quickly show proof of concept) process development for sIPV based on the established IPV production process, the subsequent manufacturing of clinical lots, their stability and pre-clinical studies. This work resulted in a vaccine that has recently been tested in the clinic (phase I/IIa) [19,20].

Methods

Ethics statements regarding animal studies

The abnormal toxicity study in suckling mice and guinea pigs and immunogenicity tests in rats used in this study were agreed upon by the Committee on Animal Experimentation of the Netherlands Vaccine Institute (Bilthoven, the Netherlands) (Study Permit numbers AAP 201000262, 201000302, 201000303, 2010000304, 2010000305, 2010000306, 2010000307, 201000310, 201100030, 201100054, 201100056, 201100101, 201100151, 201100170, 201100195, 201100214, 201100289, 201100345, 201200137, 201200154, 201200227 and 201200262). Animal handling in this study was carried out in accordance with relevant Dutch national legislation, including the 1997 Dutch Act on Animal Experimentation.

The protocol for the toxicity study in rabbits was reviewed and approved by the Animal Welfare Officer and Ethical Committee of WIL Research Europe B.V. (former name:

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Table 1. Biosafety and viral safety testing of Sabin PV master (MS) and working (WS) seedlots.

Seedlot	Virus titer (Log_{10} $CCID_{50}$ mL^{-1})	Monkey Neurovirulence[1]	MAPREC[2]	RCT40[1]	Extraneous agents/Viral safety[3]
MS PV type 1	8.85	Not determined	Conform	Conform	Conform
MS PV type 2	7.62	Not determined	Conform	Conform	Conform
MS PV type 3	8.23	Not determined	Conform	Conform	Conform
WS PV type 1	8.90	Conform	Conform	Conform	Conform
WS PV type 2	7.85	Conform	Conform	Conform	Conform
WS PV type 3	8.45	Conform	Conform	Conform	Conform

[1] Reproductive Capacity at 40°C Temperature (RCT40) and Monkey Neurovirulence: Tests and requirements according to WHO recommendations for OPV [24]. [2] Mutant Analysis by PCR and Restriction Enzyme Cleavage (MAPREC): Test and requirements according to new WHO recommendations for OPV [25]. [3] Test and requirements according to the European Pharmacopoeia [31,43].

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NOTOX B.V.) as required by the Dutch Act on Animal Experimentation (Study Permit Numbers: DEC 08-48 and 10-18). The OECD guidance document on humane endpoints (ENV/JM/MONO/ 2000/7) is applicable for all animal studies carried out at WIL Research Europe B.V.. No distress or discomfort was noted during this study.

Lab scale experiments

Upstream processing. Vero cells obtained from WHO (10-87) originally derived from ATCC (CCL-81) were used as host for PV production. Sabin PV type 1 (LSc 2ab KP_2), Sabin PV type 2 (P712 Ch2ab-KP_2) and Sabin PV type 3 (Lot 457-III-Pfizer) were used.

Studies on virus culture conditions were carried out in 5-L glass bioreactors (Sartorius Stedim Biotech). Cell cultures were done in EMEM supplemented with bovine serum (BS) and 3 g L^{-1} micro-carriers (Cytodex 1; GE Healthcare) with the following settings: T of 37°C, pH of 7.2 and DO (dissolved oxygen) of 50% by headspace aeration. Glucose was added daily when the concentration was below 5 mM. Prior to virus culture the media was exchanged to M199. Virus cultures conditions were: T of 32.5 or 33.5°C, pH of 7.4, DO of 25% by headspace aeration.

Downstream processing. Virus was harvested, clarified, concentrated, purified, first on size using size exclusion chromatography (SEC) and second by ion exchange chromatography (IEX), and finally inactivated as described previously [13].

Analytics. Cell counts were determined using a Nucleocounter (Chemometec). Glucose concentration was determined using a Bioprofile 100 plus (Nova Biomedical, MA). Cytopathic effects (CPE) were monitored microscopically. Virus was quantified by titer measurements ($CCID_{50}$) [21] and by a modified D-antigen ELISA [22] for in-process samples.

Clinical lots manufacturing

Cell and virus culture. Vero cells from a manufacturers working cell bank were used. Master and working seedlots were prepared from Sabin type 1 LSc 2ab KP_2 (WHO/ Beringwerke SO+1, 1976), type 2 P712 Ch2ab-KP_2 (WHO/ Behringwerke SO+1, 1976) and type 3 (Pfizer RSO1, SO+5, lot 457-III, 1963; supplied by Institute Mérieux to RIVM in 1991) [23]. Working seedlots were additionally tested for neurovirulence in monkeys (Bio Farma, Indonesia) [24] and analyzed with MAPREC (mutant analysis by PCR and restriction enzyme cleavage; NIBSC/HPA, UK) [25] and RCT40 (replicating properties 36°C- 40°C; AFSSAPS, France) [24] to assess genetic stability with respect to biosafety (Table 1).

Cell and virus culture was carried out in two 350-L (working volume) bioreactors. In short, thawed Vero cells were used to directly inoculate a 15-L fed-batch pre-culture (EMEM supplemented with BS and Cytodex 1 microcarriers) [26]. After trypsinization [26]; a 2nd pre-culture using the recirculation culture method [27] was done to have sufficient cell to inoculate two 350-L bioreactors at 0.1 × 10^6 cells mL^{-1}. After medium exchange [28], virus culture was started (M199; Multiplicity of infection (MOI)=0.01; T=32.5°C).

Purification. Virus from the two 350-L (working volume) bioreactors was harvested via a sieve (mesh 75 μm) to obtain a virus harvest free of micro-carriers [13]. Clarification was done using normal flow filtration with a Millipore POD-holder containing C0HC depth filters followed by an Express SHC 0.45/0.22 μm combination filter (Millipore) [3,13] . Concentration was done by tangential flow filtration using 100 kDa filters [13]. Purification was done by size exclusion chromatography (Sepharose CL-6B (GE Healthcare) [11,29]; elution buffer 20mM phosphate buffer pH 7.0) and ion exchange chromatography (DEAE-Sephadex A50 (GE Healthcare) [11,30]; elution buffer 20mM phosphate buffer pH7.0).

Inactivation. Purified virus was stabilized with concentrated M199 containing glycine (final conc. 5g L^{-1}). Inactivation was done following the standard method: 0.025% formaldehyde incubation for 13 days at 37°C. An intermediate filtration (0.22 μm) was performed at day 6-8 [5]. The resulting monovalent bulk was stored at 2-8°C.

Preparation of trivalent vaccine. Monovalent bulks of PV type 1, 2 and 3 were mixed to a ratio of 60:96:192 D-antigen prior to sterile filtration. The sIPV final bulk was subsequently prepared by addition (via 0.22 μm filter) of the mixed trivalent bulk and dilution buffer containing phosphate, phenoxyethanol and formaldehyde. Aluminium hydroxide adjuvanted sIPV final bulk was prepared as described above with the inclusion of the addition of sterile Alhydrogel (Brenntag) (directly) to the final bulk. Final bulks were mixed for 10 minutes prior to setting the

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Table 2. Release requirements[1] of plain and adjuvanted sIPV.

Cell culture		
Identity	Vero cells	
Mycoplasma	Absent	
Extraneous viruses	Absent	
Virus harvest		
Sterility	Absence of growth (Tryptic Soy Broth & Thioglycollate broth)	
Mycoplasma	Absent	
Extraneous viruses	Absent	
Purified virus		
Purity (ratio total protein and D-antigen)	≤0.1 µg DU^{-1}	
Sterility	As above	
Identity	PV type 1, 2 OR 3	
Residual host cell proteins	Consistent clearance	
Monovalent Bulk		
Inactivation kinetics	PV titer below detection limit after 120h	
Formaldehyde	>2 mM	
Sterility	As above	
PV identity	Contains PV type 1, 2 OR 3	
Inactivation	Full absence of active PV after 10 and 13 days	
D-antigen content	Information for calculation	
Trivalent bulk		
Inactivation	Full absence of active PV in 1,500 calculated human doses	
Sterility	As above	
D-antigen content	Information for calculation	
Final bulk	Plain	Adjuvanted
pH	6.8-7.4	6.8-7.4
Phenoxyethanol	31-42 mM	31-42 mM
Formaldehyde [2]	0.7-2.4 mM	0.7-1.3 mM
Sterility	As above	As above
D-antigen content	≥75% nominal value	≥75% nominal value
Final lot	Plain	Adjuvanted
Appearance	Bright red-orange fluid	Turbid red-orange fluid
PV identity	Contains type PV 1, 2 and 3	Contains type PV 1, 2 and 3
D-antigen content	≥75% nominal value	≥75% nominal value
Residual host cell DNA [3]	≤100 pg shd^{-1}	≤100 pg shd^{-1}
Bovin Serum Albumin [3]	≤50 ng shd^{-1}	≤50 ng shd^{-1}
Total protein	≤20 µg mL^{-1}	≤20 µg mL^{-1}
Endotoxin	≤10 IU mL^{-1}	≤10 IU mL^{-1}
Extractable volume	≥0.5 mL	≥0.5 mL
pH	6.8-7.4	6.8-7.4
Sterility	As above	As above
Free D-antigen	Not applicable	≤ 1%
Aluminium	Not applicable	0.26-0.36 mg mL^{-1}
Abnormal toxicity (in mice and guinea pigs)	No illness	No illness

[1] Most important release tests drafted for the production of the phase I clinical lots are given. It should be noted that the release criteria could change due to further product development. [2] Formaldehyde requirement is dependent on the amount of monovalent bulk used to prepare the final bulk. [3] Test is performed at an earlier stage in view of the lower detection limit.

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pH. The final lots were prepared by filling the final bulk in aliquots of 0.6-0.7g in 3 mL sterile siliconized vials using a Bausch & Ströbel ksf 1027 machine.

Analytics. During the process, sampling was done, as required for release of sIPV final lots. In Table 2 a list of the most relevant assays for release is given. A qualified QC department carried out these assays.

Cell counts were performed using a hemocytometer. Cythopathic effects (CPE) were monitored microscopically. Virus was quantified by titer measurements ($CCID_{50}$) and D-antigen ELISA [14] for release. SDS-PAGE was done using

precast 4-20% gradient gel (Pierce) with Tris-HEPES buffer (Pierce). Vero host cell protein concentrations were determined using a Vero Cell HCP ELISA kit F500 according to the manufacturer's instructions (Cygnus Technologies, NC). MAPREC and RCT40 were performed as described in WHO guidelines [24,25].

Preclinical studies - Rat immunogenicity. The rat immunogenicity was determined essentially as described previously [31,32]. In short, TOX rats (weighing 175-250g) that were screened for the absence of PV antibodies were injected intramuscularly with 0.5 mL of prepared vaccine dilutions (group size 10 per dilution). Blood samples were taken 21 days after injection. Collected sera (stored at -20°C until use) were analyzed for neutralizing antibodies. Serial dilutions (with M199) of heat inactivated sera (30 min 56°C) were prepared in a 96-wells plate and incubated with 50 µl (2×10^3 $TCID_{50}$ mL^{-1}) PV type 1 Mahoney, PV type 2 MEF-1, or PV type 3 Saukett for 3h at 35-37°C in a CO_2 incubator and subsequently stored overnight at 2-8°C. After addition of Vero cells (50 µL at 2×10^5 cells mL^{-1}) the 96-wells plates were incubated for 7 days at 35-37°C. Supernatants were discarded and cells were stained with a crystal violet solution containing 5% formaldehyde. Presence of full monolayers of Vero cells indicated a complete neutralization of the virus. The neutralization antibody score represents the highest dilution (log_2; with a test maximum of 12) where complete neutralization was observed. For comparison of sIPV with conventional IPV, the international standard PU91-01 was diluted towards the conventional IPV dose (40/8/32 DU/shd).

Preclinical studies - Toxicity study in rabbits. A repeated dose and local tolerance toxicity study followed by a two week recovery period was carried out in rabbits (NOTOX, the Netherlands) according to EMA guidelines [33]. In short, New Zealand white rabbits (group size of 16, equally distributed among sexes) were treated by intramuscular injection with 0.5 mL vaccine or placebo (vaccine without D-antigen) at day 1, 15, 29, 43 and 57). Animals were necropsied at day 60 (n=10) or day 71 (n=6). The following observations and examinations were evaluated: clinical signs (daily), skin irritation (24 and 48 hours after each administration), body weight (weekly), food consumption (twice weekly), ophthalmoscopic examination (during pretest, end of treatment and end of recovery), rectal body temperature (during pretest, prior to each dosing and approximately 2 hours after dosing), clinical pathology (Pretest, Days 4, 57, 60 and 71), macroscopy at termination and organ weights and histopathology on a selection of tissues.

Statistical analysis

Two sided Student t-tests were performed with α=0.05. Numbers are given as means with standard deviations.

To determine the regression line slopes, no weighted regression was used. This was chosen based on the use of medians when concerning animal tests in contrast to the use of the means. In addition, the observed standard deviation from the median did not increase with increasing values. The significance (95% confidence) of the slopes was tested using an extra sum of squares F-test with the null hypothesis being a horizontal line (i.e. slope=0) (Graphad Prism 6 for Windows).

Results

Drafting product and process specifications

The specifications (Table 2) for the release and control of sIPV and aluminium hydroxide adjuvanted sIPV were drafted based on WHO [5] and EP monograph [31] for IPV manufacturing. Some product requirements, like formaldehyde content and pH, were based on the available IPV experience. Requirements related to the adjuvation with aluminium hydroxide were set after initial research [34]. The WHO OPV guidelines [24] were taken into account to assess the Sabin PV genetic stability with respect to biosafety, i.e. temperature sensitivity and revertants.

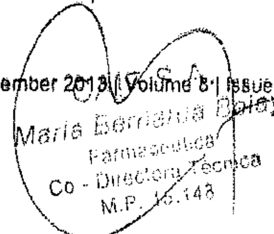
Process development prior to manufacturing of clinical lots

In view of the relatively short timelines in the polio eradication program it was chosen to prepare a sIPV with limited process development time prior to production of clinical lots. Process development therefore focused on Sabin strain specific adaptations like MOI and virus cultivation temperature and chromatography (discussed below). In addition, a disposable clarification unit was introduced to replace the Celite cake for depth-filtration. On all other aspects the production process was similar to conventional IPV manufacturing.

Selection of MOI and virus cultivation temperature. In OPV manufacturing, the virus cultivation temperature for Sabin PV is lower (at a maximum of 35°C [24]) than the temperature used for wild-type PV in conventional IPV manufacturing (36-37°C) [18,35]. This lower temperature is required to ensure the temperature sensitivity of the Sabin PV and minimize revertants to ensure a safe OPV. Although here an inactivated product has been developed, manufacturing itself should be biosafe and one of the prerequisites was to ensure the safety of the prepared virus harvest with respect to revertants of Sabin PV.

The effects of virus culture temperature and multiplicity of infection (MOI) on the virus culture yields and culture time were assessed using PV type 2. No differences in virus yields were observed when the MOI was decreased from 0.1 to 0.01. Decreasing the temperature from 33.5°C to 32.5°C, had a negative effect on virus yields. Virus titers were 7.7 ± 0.1 (n=3) and 7.2 ± 0.1 (n=3) Log_{10} $CCID_{50}$ mL^{-1} for cultures at respectively 33.5°C and 32.5°C. D-antigen (a measure for immunogenic virus) concentrations were 25 ± 3 (at 33.5°C) and 11 ± 5 DU mL^{-1} (at 32.5°C). Under all tested conditions virus culture was complete within 4 days, i.e. cytopathic effect (CPE) >90% and both virus titers and D-antigen (a measure for immunogenic virus) concentrations remained constant. Despite the lower yields at 32.5°C, this cultivation temperature was selected for preparing the virus seeds and clinical trial material. This choice was made to minimize the risk of PV revertants. Since no difference was observed when using a lower MOI, an MOI of 0.01 was used as it is preferred since smaller amounts of virus working seedlots will be needed.

Chromatography resin selection and buffer strength. Initial process development was done to confirm the



use of resins and procedures available for wild-type PV. The present SEC resin and procedure were applicable for use with Sabin PV (data not shown). For IEX a choice between two validated resins needed to be made. Both DEAE Sephadex A-50 [30] and DEAE Sepharose Fast Flow [13] have been used for purification of PV. In both cases impurities are captured while Salk PV flows through. Initial studies using Sabin PV Type 1 showed some non-specific binding of the PV to DEAE Sepharose Fast Flow; this was confirmed for Sabin PV Type 2. The alternative resin DEAE Sephadex A50 allowed efficient separation of Sabin PV type 1, 2 and 3.

During the proof of principle study [23] in which OPV bulks were obtained to generate IPV, a precipitate was noticed during inactivation. Analysis showed that this precipitate was a phosphate based precipitate without product. The main source of the phosphate was traced back to the purification process where a 40mM phosphate buffer was used during chromatography (both SEC and IEX). Application of a weaker phosphate buffer (20mM) and the impact on product elution and inactivation was assessed. Product elution profiles in SEC and IEX using a 20mM phosphate buffer were comparable with those obtained after eluting with a 40mM phosphate buffer. Inactivation of virus eluted with 20mM phosphate was comparable while precipitates were absent.

Manufacturing of clinical lots

Preparation of virus seed. Sabin PV strains closest to the Sabin original strains (PV T1: SO+1 Behringwerke 1976; PV T2: SO+1 Behringwerke 1976; and PV T3: RSO+1 Pfizer 1963) were used to produce new virus master seedlots at 10-L scale. The virus working seedlots were produced at 350-L scale [3]. These seeds were tested for neurovirulence using the monkey neurovirulence test [24]. However, due to limited global test capacity and to minimize costs it was chosen to only test the working seedlots. It was argued that the working seeds represent, on a worst case base, the quality of the master seed with respect to neurovirulence. Next to passing the neurovirulence test, the master and working seedlots also passed the tests for extraneous agents, RCT40 (reproductive capacity at 40°C temperature) and MAPREC (Mutant Analysis by PCR and Restriction Enzyme Cleavage) (Table 1).

Upstream processing. The preparation of sIPV was done in a physically separated production area in the established cGMP facilities for conventional IPV manufacturing. For each virus type two monovalent bulks were prepared. Vero cell culture was carried out in twin 350-L bioreactors [12]. In Figure 2A, the average growth curve of the 12 cultures (6 bulks in twin bioreactors) at 350-L scale is given. Cell culture was started at an inoculation density of 0.1 × 10^6 cells mL^{-1}. Cells grew adherent to micro-carriers (average growth rate 0.025 h^{-1}) to reach 1.1 × 10^6 cells mL^{-1} after 4 days when the micro-carriers were covered by a confluent layer of cells. At this point, the bovine serum containing medium was exchanged with serum free virus culture media. Subsequently, cells were infected with Sabin PV. Virus culture proceeded comparably and was independent of the virus type used for infection. Cell lysis was complete after 4-5 days as was determined based on the CPE observed microscopically (Figure 2B). Virus yields were based on virus titers (Figure 2C) and D-antigen (Figure 2D). While comparable virus titers were observed for the production of Sabin PV type 1 and 3, the yields for Sabin PV type 2 were significantly lower (2-tailed t-test; α=0.05 p=0.0043). D-antigen yields are not comparable between virus types as they are type (and antibody) specific [36]. From Figure 2C&D it was concluded that virus cultures were reproducible. The virus harvests were negative for revertants of Sabin PV as analyzed by RCT40 and MAPREC.

Downstream processing. Virus from the twin bioreactors was harvested and pooled prior to purification. Harvested virus was first clarified using normal flow filtration (NFF), which resulted in a decrease in fluid turbidity from 54 ± 6 NTU (Nephelometric Turbidity Unit) to 0.4 ± 0.4 NTU (n=6; determined mid-processing). The clarified virus was subsequently concentrated from approx. 700L to 1L using tangential flow filtration (TFF). Product recoveries, based on D-antigen units (DU), during the filtration steps were 90% ± 3% and 68% ± 11% for respectively NFF and TFF (Table 3).

The concentrated product was purified using 2-step chromatography starting with SEC. In Figure 3A a typical SEC elution pattern for Sabin PV is given. The 1st peak contains mostly large cell components. PV is found in the 2nd peak as is illustrated by SDS-PAGE (Figure 3B) where the presence of the viral proteins is more pronounced when purification with SEC was done. An average D-antigen recovery of 68% ± 11% was found for SEC (Table 3).

Subsequently the negatively charged molecules, like nucleic acids and proteins, were removed using IEX chromatography. PV should not interact with the matrix as was the case for Sabin PV type 1 and 3, where a plug flow was observed (Figure 3C). However, Sabin PV type 2 showed some interaction with the matrix as is apparent from the chromatogram (Figure 3C). The presence of the plug flow type chromatogram for Sabin PV type 1 and 3 allowed collection of the PV after IEX without major losses. Sabin PV type 1 and 3 were collected with 99.5% D-antigen recovery (Table 3). Relatively high losses (72%) were observed during IEX for Sabin PV type 2 (Table 3). The efficiency of the purification is illustrated by the SDS-PAGE in Figure 3B. After IEX the viral proteins are clearly purified from the other protein present after SEC. Removal of impurities was also shown for Vero host cell proteins and host cell DNA (Figure 3D). After IEX host cell protein concentrations were below 0.3 µg mL^{-1}, corresponding to an over 1,000 times removal. Host cell DNA concentrations were below the detection limit of 78.13 pg mL^{-1} which is below the maximum level allowed in a single human dose (shd) (Table 2).

After IEX the Sabin PV was inactivated during a 13-day incubation period with formaldehyde. PV was inactivated rapidly, i.e. within 4 days, as shown in Figure 3E. After 6 to 8 days an intermediate filtration step was carried out to remove possible aggregates and ensure full inactivation. After inactivation a large variation in D-antigen recovery was observed, especially for Sabin PV type 3. Overall recoveries ranged from acceptable (in conventional IPV manufacturing on average approximately 40% for all three sub-types [13,37]), for Sabin PV type 1 (at 40%) to very low, with respect to future

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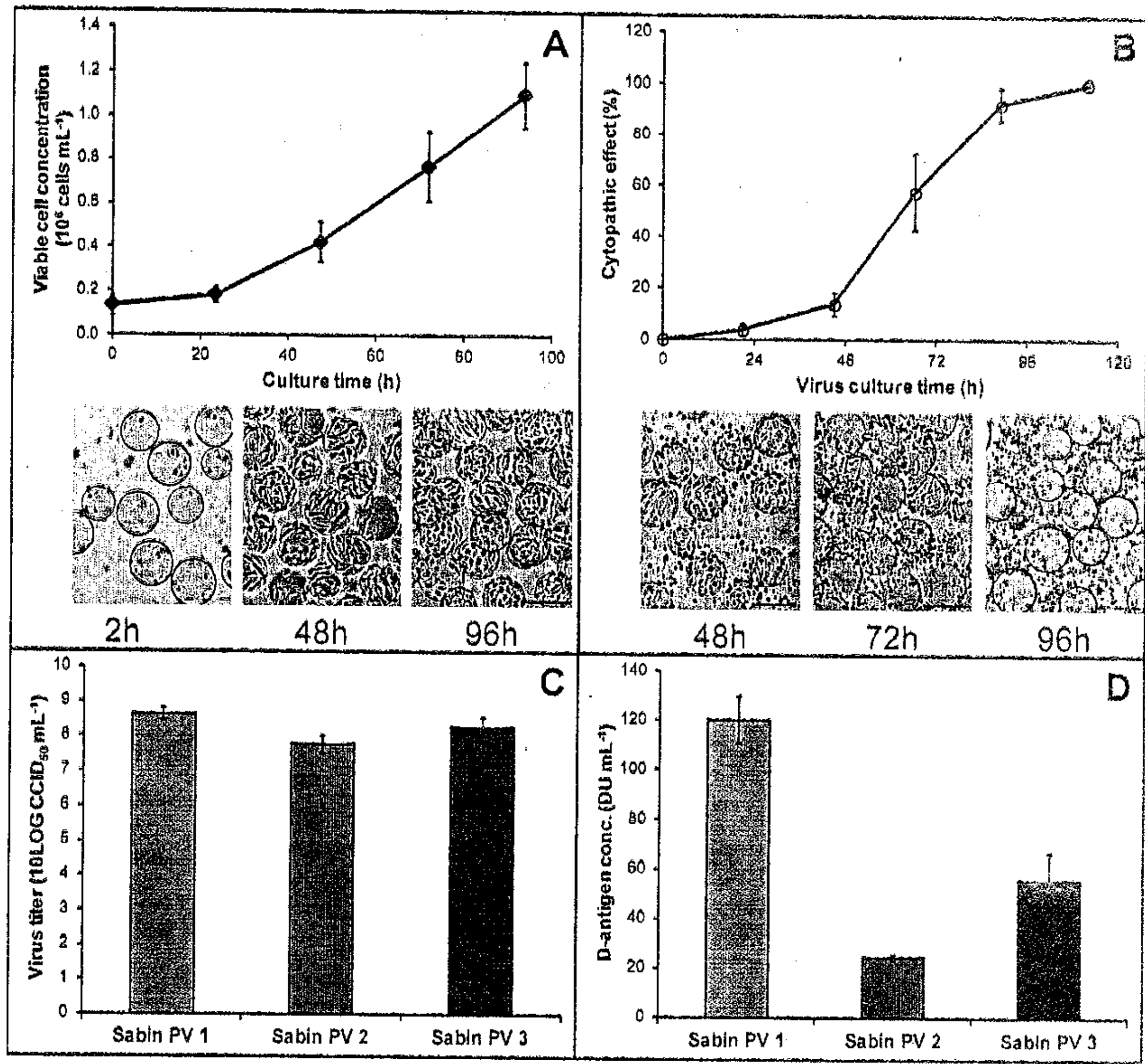

Figure 2. Cell and virus culture. Panel A shows the average Vero cell growth curve (n=12; error bars represent SD) in 350-L bioreactors. Photographs are light microscopy images (size bar 200 µm). Panel B shows the average (of the three subtypes) Vero cell death during virus culture determined microscopically (n=12; error bars represent SD). Photographs show corresponding images. Panel C shows average virus titers for Sabin PV type 1, 2 and 3 (n=4; error bars represent SD). Panel D shows average D-antigen concentrations after virus culture for Sabin PV type 1, 2 and 3 (n=4; error bars represent SD).

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cost competitive processing, for PV type 2 (at Table 3). The obtained monovalent bulks met all release criteria and were stored at 4°C prior to mixing for formulation.

Formulation. Monovalent bulks were mixed to a trivalent bulk (Sabin PV type 1-2-3) in a ratio of 60-96-192 DU mL^{-1} prior to formulation to a final bulk. Different final bulks were prepared. Plain sIPV was prepared in different final concentrations of D-antigen to be able to test low (5-8-16 DU shd^{-1}) middle (10-16-32 DU shd^{-1}) and high (20-32-64 DU shd^{-1}) dosages in (non-)clinical studies. Aluminium ($Al(OH)_3$) adjuvanted vaccine was mixed at 2-fold lower D-antigen values being: low (2.5-4-8 DU shd^{-1}), middle (5-8-16 DU shd^{-1}) and high (10-16-32 DU shd^{-1}). Vaccine was filled in vials as 0.5 mL per single human dose.

Pre-clinical studies

The immunogenicities in terms of the capacity to induce virus neutralizing antibody titers (VNT) against the wild-type PVs (PV Type 1 Mahoney, PV Type 2 MEF-1 and PV Type 3 Saukett) of the six differently formulated vaccines were determined in rats

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Table 3. Product recovery during processing of two batches for each serotype.

Virus subtype	Harvest	Clarification	Concentration	SEC[2]	IEX[3]	Inactivation	Overall[1]
Sabin PV type 1	100%	86%	82%	82%	90%	84%	38%
	100%	89%	73%	67%	107%	85%	41%
Sabin PV type 2	100%	92%	77%	69%	20%	60%	<15%
	100%	96%	54%	51%	35%	64%	<15%
Sabin PV type 3	100%	91%	75%	75%	87%	36%	18%
	100%	88%	70%	83%	114%	72%	24%

Losses due to sampling were not considered for recovery calculations of individual unit operations. The overall product recovery includes losses due to sampling for in-process and release tests as well as sampling for research purposes.

[1] The overall DSP yield was calculated by dividing the amount of D-antigen units of the monovalent bulk by the amount of D-antigen units from the harvest. [2] Size Exclusion Chromatography [3] Ion Exchange Chromatography

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[31,32]. High VNT against wild-type viruses were observed for all prepared formulations (i.e. 0.5 mL of high, middle and low DU). The maximum VNT for PV type 1 was lower than for PV type 2 and 3 (Figure 4A-C). For all types, the VNT increased with the dose and the addition of aluminium as adjuvant had a positive effect. For PV type 2 this effect was larger than for PV type 1 and PV type 3. Compared to conventional IPV, immunization of rats with sIPV resulted in comparable wild-type VNTs for PV type 1 and 3. Lower VNTs were found for PV type 2 when immunized with sIPV, however the levels of antibodies raised are very high (>8 $\log_2$) (Figure 4D-F). These data suggest that sIPV may be able to raise sufficient protective antibodies against all PV sub-types in humans, where the threshold for protection is 3 $\log_2$ [38] and thereby would be non-inferior to conventional IPV.

Stability of the clinical lots over a period of 24 months was assessed based on immunogenicity in rats, D-antigen concentration, amount of free D-antigen (in case of adjuvanted vaccine) and more general parameters like sterility, appearance and pH. Vaccine stability with respect to immunogenicity in rats is illustrated in Figure 5A-D. Based on the regression line slopes (derived from Figure 5A) and their 95% confidence intervals (Figure 5B) it was concluded that all formulated clinical lots were stable with respect to immunogenicity in rats (null hypothesis slope=0; α=0.05; result $p>0.05$).

In a similar way, the stability of the D-antigen content in the formulated clinical lots was reviewed. Slopes and their 95% confidence intervals of the regression lines (as illustrated in Figure 5C) were calculated. The measurement of D-antigen in aluminium adjuvanted formulations was difficult as the D-antigen needed to be desorbed from the aluminium prior to performing the D-antigen ELISA. This hurdle is illustrated by the larger confidence intervals found for the regression lines for D-antigen stability for the adjuvanted vaccines (Figure 5D). As a result no conclusions with respect to D-antigen stability could be drawn for the adjuvanted vaccine. Stability regarding the D-antigen content of the non-adjuvanted (plain) vaccine was good (null hypothesis slope =0; α=0.05; result $p>0.05$).

A repeated dose and local tolerance toxicity study in rabbits was conducted. Highest dose plain and adjuvanted sIPV were compared to a placebo and conventional licensed IPV. Some enlargement in local lymph nodes was found in all vaccine treated groups. Generally, minimal to mild inflammation was observed microscopically at the injection sites of all groups and could be attributed to the injection trauma. More intense inflammation was shown in the adjuvanted sIPV group, which was, in contrast to the other groups, not diminished after the two week recovery period. This was solely attributed to the apparent persistence of the adjuvant. A longer recovery period should have been chosen. The changes at the injection sites as well as the changes noted in the local lymph nodes are common findings in intramuscular vaccine studies meaning that the vaccines are safe to use in clinical trials.

Discussion

The polio eradication program strives to a switch from OPV to sIPV and currently at least one dose of IPV is recommended [39]. In view of the relatively short timelines in the polio eradication program it was chosen to prepare a sIPV with limited process development time prior to production of clinical lots. Based on the existing large scale IPV manufacturing process development of sIPV was achieved. Main operating differences were related to the observed precipitate during inactivation, intrinsic virus properties resulting in adjustments in tests (i.e. aluminium desorption prior to D-antigen quantification) and limits required with respect to biosafety (i.e. virus culture temperature). The final product met quality criteria and could be released for testing in the clinical phase I/IIa studies in adults and infants to show safety and proof of principle.

Although a sIPV with required immunogenicity and purity could be produced, the purification yields with respect to Sabin PV type 2 were very low. These low levels will not result in a cost competitive IPV product. However, in light of the polio eradication program, and to pursue the fast implementation of worldwide sIPV manufacturing, sIPV production was continued despite the low type 2 yields. In this way it could be illustrated whether such a product would be comparable or better for polio vaccination compared to the conventional IPV.

The next step in the project is to transfer the manufacturing knowledge to current vaccine manufacturers in low- and middle income countries to replace the OPV production with sIPV

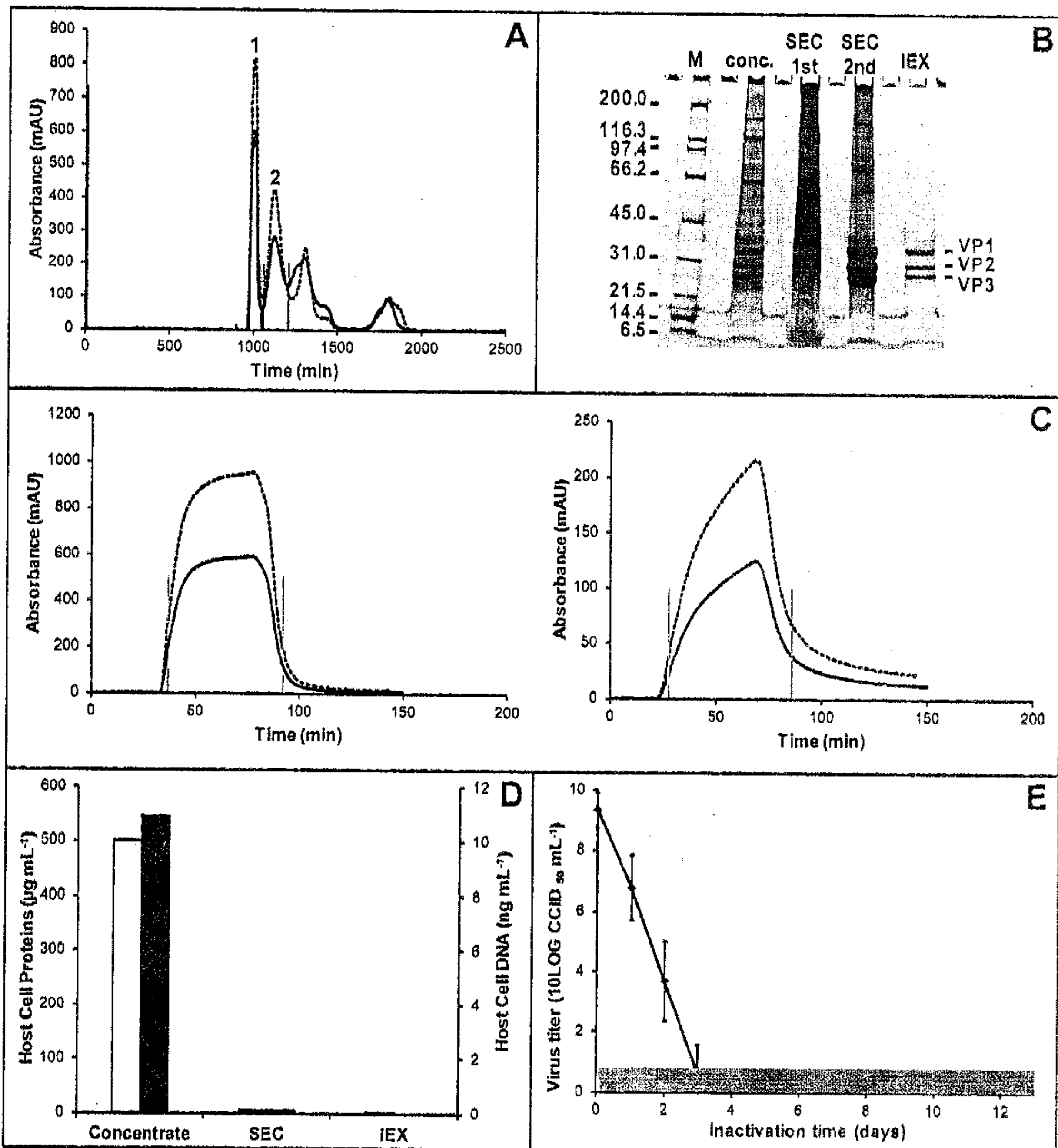

Figure 3. Purification of Sabin PV. Panel A depicts a SEC chromatogram of Sabin PV type 1. The 1st peak contained mostly large cell components; the 2nd peak contained the majority of PV, following peaks consist of smaller components. Panel B shows a SDS-PAGE (4-20% gel); lanes represent (from left to right) the marker, the concentrated product, followed by the 1st and 2nd fraction of SEC and finally the IEX purified PV. Panel C shows chromatograms of Sabin PV type 1 (left) and Sabin PV type 2 (right) IEX purification. Panel D shows host cell protein (open) and DNA (solid) impurities. Panel E depicts the inactivation of PV, the gray area indicates the lower detection limit. In chromatograms A and C, the dotted and solid lines represent absorbance at respectively 254nm and 280nm. Gray dotted lines indicate peak fractioning.
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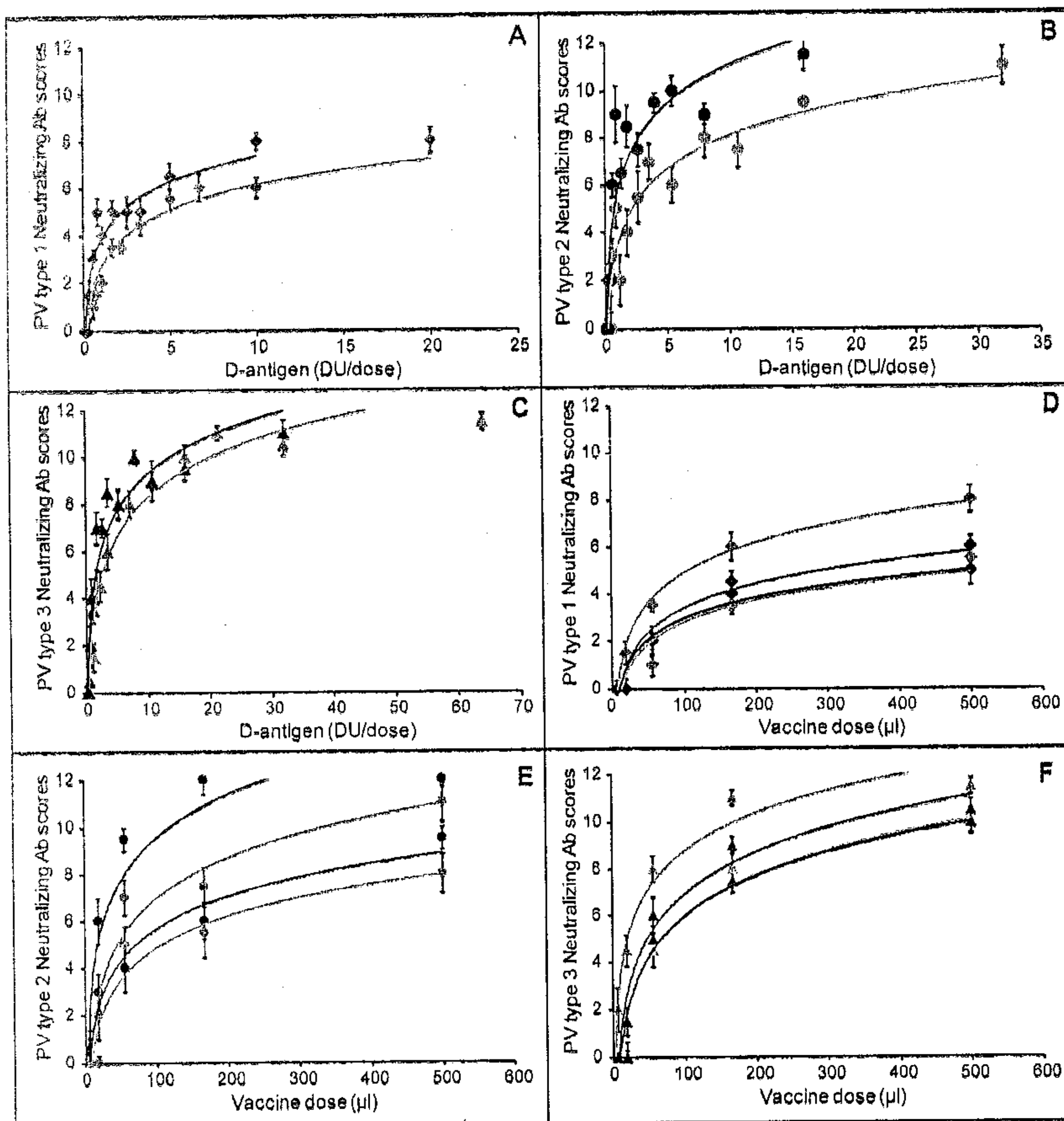

Figure 4. Rat Immunogenicity (VNT against wild-type viruses). Panel A, B and C: VNT (log_2 titer) to immunization with plain sIPV (blue) and adjuvanted sIPV (red) for PV type 1, 2 and 3 respectively; Panel D, E and F: VNT of plain sIPV 20/32/64 (light blue), 10/16/32 (red), 5/8/16 (green) and plain IPV 40/8/32 (dark blue) for PV type 1, 2 and 3 respectively. Error bars in panel A-F depict standard deviation of the median (n=10 rats).

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production [3]. The presented manufacturing process is being optimized in parallel with technology transfer. As product registration for market authorization at local authorities will require local clinical studies, necessary process optimizations for an economically feasible product can be implemented prior to this stage.

Worldwide efforts in the development of sIPV have recently resulted in market authorization for two vaccines containing sIPV in Japan [40]. Further, the Institute of Medical Biology,

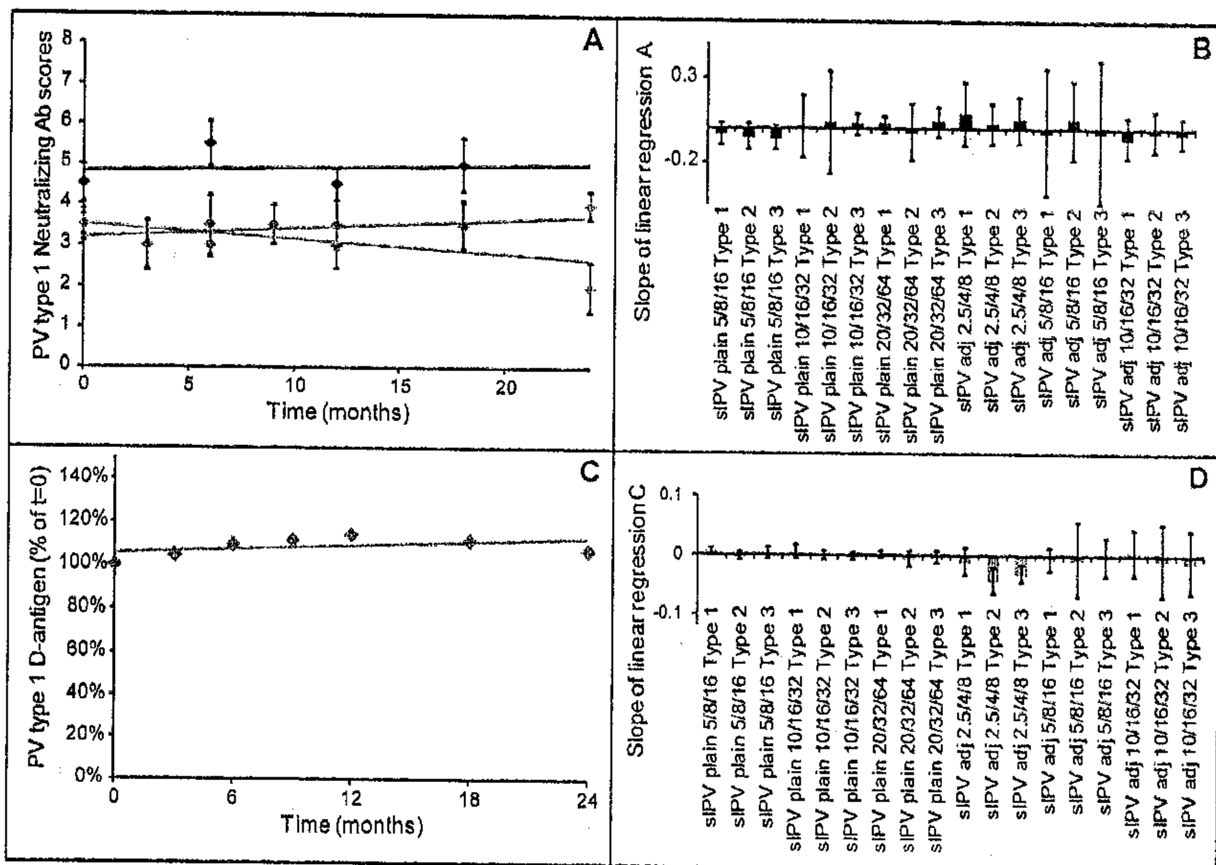

Figure 5. Stability of sIPV. Panel A: PV type 1 VNT of plain sIPV 20/32/64 (blue), 10/16/32 (red), 5/8/16 (green) in time, error bars depict standard deviation of the median (n=10 rats); Panel B: slopes of linear regression lines determined for stability based on rat immunogenicity as illustrated in panel A, error bars depict 95% confidence interval; Panel C: Stability of PV type 1 D-antigen of sIPV 20/32/64; Panel D: slopes of linear regression lines determined for stability based on D-antigen as illustrated in panel C, error bars depict 95% confidence interval.

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Chinese Academy of Medical Sciences (Kunming, China) [41] is currently performing clinical phase III studies [42]. This parallel development of sIPV allows a solid base for future IPV availability and minimization of risks with respect to biosafety during manufacturing.

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Author Contributions

Conceived and designed the experiments: YT AO MO WB. Performed the experiments: YT AO MO. Analyzed the data: YT. Wrote the manuscript: YT. Supervision of the work: WB. Conceptual advice and manuscript editing: LvdP RW WB.

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