Immune Response in Guinea Pigs and Monkeys to the Individual Components of a Combined Diphtheria-Pertussis-Tetanus Antigen Plus Poliomyelitis Vaccine

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In two groups of laboratory animals injected with combined antigens, each antigen stimulated its own antibody. How human beings will react can be determined ultimately only by tests on Homo sapiens himself.

The use of combined multiple antigens for immunization against several diseases is not new. Castellani [1] combined typhoid, para A, and para B antigens (TAB) in 1905, and subsequently reported on the addition of cholera vaccine, later, plague, and finally mellitensis vaccine to TAB making a six-antigen preparation. In a review article, Ramon [2] refers to his work with Zoeller in 1926 in which they used "mixed vaccines" which included TAB and tetanus toxoid, TAB and diphtheria toxoid, and also TAB with both toxoids. They found that the immunizing activity of toxoid was increased when used in a mixed vaccine. Jones and Moss [3] found no interference with the specific immunologic response to combined diphtheria and tetanus toxoids, and wide acceptance of this product is suggested by publications during the early forties, as in the committee report by Bunney.[4] Experiments in animals with diphtheria toxoid and pertussis vaccine were reported by a number of authors including Schutze,[5] Mathieson,[6] and Greenberg and Fleming.[7]

The results encouraged trials in children and a number of workers soon confirmed the good serologic response to both antigens and, in some instances, pointed out that the efficacy of the toxoid was enhanced when mixed with pertussis vaccine. In addition to serologic response, Kendrick [8] studied actual protection against pertussis in children previously vaccinated with combined diphtheria toxoid and pertussis vaccine, and found it to be of the same order as in a group of children injected with pertussis vaccine alone. More recently, studies of triple diphtheria-pertussis-tetanus (DPT) vaccine have included those by di Sant' Agnese,[9] McComb and Trafton,[10] Sauer and Tucker,[11] Peterson and Christie,[12] and Volk, [13] and the results of its use have been discussed by Ipsen.[14] In general there has been good response to each of the antigens in the combination.

Since there are established programs

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for the immunization of infants, it seemed reasonable to assume that if poliomyelitis vaccine proved effective in field trials, a logical question would concern the possibility of combining it with a currently acceptable product, such as triple DPT. This question assumes increasing importance with the recent demonstration of the effectiveness of poliomyelitis vaccine in children by Francis [15, 16] in the evaluation study under his direction, by Salk [17, 18] and more recently, in young infants, by Brown and Smith.[19] Additional information must be obtained before the application of such a combined product in the active immunization of children; however, much can be learned from experiments with laboratory animals. The present report describes studies of the serologic response of guinea pigs and monkeys to a combined DPT-poliomyelitis vaccine in comparison with DPT alone and poliomyelitis vaccine alone.

Experimental Methods

Vaccines

The triple diphtheria-pertussis-tetanus (DPT) vaccine used in this study was furnished by the Michigan Department of Health, Lots B-1063 (alum precipitated) and B-1117 (aluminum phosphate adsorbed). Both lots were in current use in Michigan for immunization of children with a dosage of 0.5 ml given three times. Poliomyelitis vaccine trivalent, Reference A, was furnished by Dr. Jonas E. Salk and had served as a reference lot in different laboratories.

Before combination of the triple and poliomyelitis vaccines, the DPT was treated with versene to avoid a possible subsequent deleterious effect related to the presence of merthiolate on the poliomyelitis component—an effect which has been previously described.[20, 21] This was done by adding 0.07 ml of 1:10 versene to each 10 ml quantity of triple DPT vaccine. After two hours at room temperature, DPT and poliomyelitis vaccines were mixed in equal volumes and stored at 4° C in the refrigerator until used. For convenience this combined antigen is designated DPT-P.

Tests for Antibodies

Pertussis agglutinins were determined by mixing equal 0.1 ml quantities of pertussis antigen containing 20 billion organisms per milliliter (determined photometrically) and twofold dilutions of the serum to be tested. These mixtures were shaken for three minutes and incubated at 37° C for one hour. After the addition of 0.5 ml of physiologic salt solution to each tube, readings were made immediately in terms of visible agglutination and the end point taken as the highest dilution of serum in which definite agglutination was observed.

Neutralizing antibodies for poliomyelitis were determined by making original serum dilutions of 1:4, 1:8, 1:16, 1:64, 1:256, and 1:1,024 and mixing with equal quantities, respectively, of the three immunologic types of virus diluted to represent 100 tissue culture doses in the final inoculum. After incubation for one hour at room temperature 0.5 ml of each virus-serum mixture was added to stationary tubes of Hela cells containing 0.25 ml of maintenance solution. Appropriate virus titration and tissue and type-specific serum controls accompanied each test. The tubes were incubated at 37° C and beginning at three days were examined under the microscope for the cytopathogenic effect of the virus. The highest dilution of serum completely protecting against virus activity was taken as the neutralizing titer.

Units of circulating diphtheria and tetanus antitoxin were determined by procedures commonly in use in biological laboratories. Suitable dilutions of each serum were combined with an Lr/1,000 dose of toxin and 0.1 ml of

each mixture was injected intradermally into rabbits. After 72 hours the dermal reaction was compared with that produced by 0.001 unit of standard antitoxin combined with the same dose of toxin. For tetanus antitoxin, tested also at the 1/1,000 level, dilutions of each serum were combined with L+/1,000 dose of toxin and 0.2 ml injected subcutaneously in mice, and the time of death compared with that of mice injected with 0.001 unit standard antitoxin combined with the same dose of toxin.

Experimental Results

Antigenicity Tests of the Vaccines Under Study

While the triple DPT used in the present experiments had met with the usual Minimum Requirements for release by the National Institutes of Health, it was retested under conditions of the experiments, that is, after treatment with versene and after mixture with poliomyelitis vaccine.

Pertussis Mouse Protection Tests—The triple DPT and DPT-P vaccines were tested in mice, using intracerebral challenge, for relative potency of the pertussis component, at the Michigan Department of Health Branch Laboratory in Grand Rapids. The 50 per cent end points for the immunizing dose (ImD/50) of the vaccines, expressed in terms of millions of organisms, were as follows: Reference vaccine (pertussis only), 420; DPT, 620; DPT-P, 420; and poliomyelitis vaccine alone, no protection. The results indicated a similar response to the pertussis component whether given in DPT or in the combined DPT-P.

Diphtheria and Tetanus Antitoxin—Triple DPT, Lot B-1117 and DPT-P vaccines were tested for stimulation of diphtheria and tetanus antitoxin in guinea pigs by the usual procedures of NIH Minimum Requirements. Eight animals were injected once with 1.0 ml amounts of each vaccine, and the results of antitoxin titrations in units per ml of each pool were:

	Units	
	Diphtheria	Tetanus
DPT + saline	5	8
DPT + versene	4	8
DPT + versene + poliomyelitis	2	5

On the basis of this one experiment in guinea pigs the antigens would have passed the usual requirements for antigenicity.

Experiments with Guinea Pigs

Three groups of eight guinea pigs each were given intramuscular injections as follows: Group I, DPT (MDH, Lot B-1063); Group II, Polio; and Group III, DPT-P vaccine. In each instance, the volume of the inoculum was 0.5 ml and the quantity of each component was the same whether given separately or in combination with the other. The animals were given serial numbers in groups I, II, and III, respectively, of 11–18, 21–28, and 61–68. Any missing numbers in these series in the tabulations indicate animals that died during the course of the experiments and therefore the comparative data were not complete. The first two injections, two weeks apart, were considered the primary course; the third injection eight weeks later, a secondary or "booster" stimulus. The animals were all bled two weeks after the second injection and again two weeks after the third.

Results

Pertussis—The results of pertussis agglutination tests with guinea pig sera are shown in Table 1. It will be seen that two weeks after the second injection there was a rather uniform response among the guinea pigs vaccinated with DPT and the titers increased appreciably following the third injection, or secondary stimulus. As expected, no agglutinins were detected in those animals that received only poliomyelitis vaccine.

Table 1—Pertussis Agglutinins in Guinea Pigs

Vaccine Group	Guinea Pig No.	Agglutination Titers Two Weeks After 2nd Injection	Agglutination Titers Two Weeks After 3rd Injection
I. Triple (DPT)	12	128 *	1,024
	13	256	512
	14	64	1,024
	15	256	1,024
	17	256	2,048
	GM ‡	180	1,024
II. Polio	21 to 28	— †	—
III. Combined Triple Plus Polio (DPT-P)	61	128	1,024
	62	256	2,048
	63	128	2,048
	64	256	1,024
	67	256	2,048
	68	256	1,024
	GM	218	1,536

* Titers are expressed as reciprocals of the highest final serum dilutions.
† — = negative at 1:8, lowest dilution tested.
‡ GM = geometric mean, calculated as arithmetic mean of logs of titers, then expressed as titers.

However, in those animals that received combined DPT-P the pertussis agglutinin response at both bleedings was at least as good as following DPT alone. This is illustrated graphically in Figure 1, on the basis of geometric mean titers.

Poliomyelitis—The poliomyelitis response of these same guinea pigs is shown in Table 2. The control group receiving triple DPT vaccine alone failed to develop poliomyelitis antibodies. In those animals that received poliomyelitis vaccine alone, the response to the Type II component following the second injection was surprisingly good. The response to Types I and III, however, was weak. On the other hand, the antibody levels observed after the third injection were much higher for all three immunologic types.

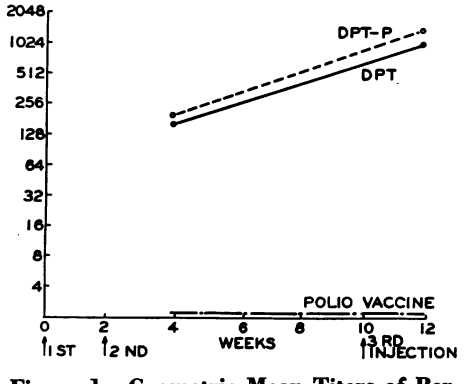

Figure 1—Geometric Mean Titers of Pertussis Agglutinins in Guinea Pigs Bled Two Weeks Following Second and Third Injections of Vaccines.

The response to the poliomyelitis component of the combined DPT-P followed the same general pattern although the levels attained were much lower. The relationship of the geometric mean titers is shown in Figure 2, illustrating the consistently higher levels obtained with poliomyelitis vaccine alone.

Diphtheria and Tetanus—The diphtheria and tetanus antitoxin levels two weeks after the second injections are summarized in Table 3. These tests show a response to diphtheria and tetanus toxoids whether given in DPT or in combined DPT-P. Because of large

variations in response among individual animals of one group the differences between groups cannot be considered of significance.

Experiments in Monkeys

Three groups of three cynomolgous monkeys were given intramuscular injections of DPT (Lot B-1117), Polio, and DPT-P vaccines, respectively. In each instance, the vaccine was given in a volume of 1 ml and the quantity of each component was the same whether given separately or in combination. The first two injections were given at an interval of two weeks; the third, seven weeks later; and the fourth, six weeks after the third. All animals were bled by cardiac puncture before the first in-

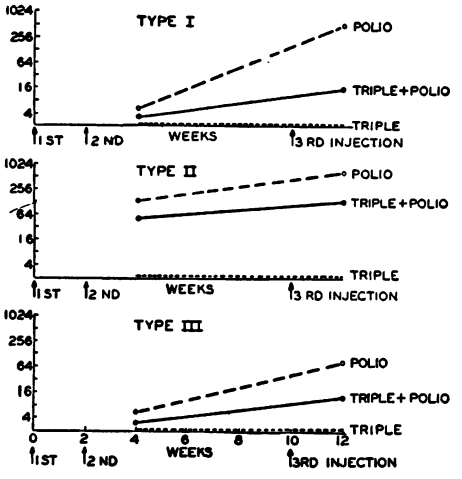

Figure 2—Geometric Mean Poliomyelitis Antibody Titers in Guinea Pigs Two Weeks Following Primary and Secondary Vaccination.

Table 2—Poliomyelitis Antibodies in Guinea Pigs

Vaccine Group	Guinea Pig No.	Titers After Second and Third Injections, by Virus Type					
		Type I		Type II		Type III	
		2nd	3rd	2nd	3rd	2nd	3rd
I. Triple (DPT)	12	—*	—	—	—	—	—
	13	—	—	—	—	—	—
	14	—	—	—	—	—	—
	15	—	—	—	—	—	—
	17	—	—	—	—	—	—
II. Polio	21	8	nd	64	nd	4	nd
	22	16	1,024	64	1,024	8	1,024
	23	8	64	1,024	64	8	16
	24	—	1,024	1,024	1,024	4	64
	25	4	1,024	64	1,024	16	256
	26	—	256	64	1,024	—	16
	28	4	1,024	64	1,024	4	64
	GM†	5	512	141	666	6	83
III. Combined Triple Plus Polio (DPT-P)	61	4	8	64	64	—	16
	62	8	256	64	256	—	8
	63	—	4	16	64	—	8
	64	4	64	64	256	—	4
	67	16	—	64	256	4	64
	68	—	16	256	64	4	8
	GM	3	16	54	128	<4	12

* — = negative at lowest dilution tested (<4).
† GM = geometric mean.
nd = not done.

Table 3—Diphtheria and Tetanus Antitoxin Levels in Guinea Pigs Two Weeks After Second Injection of Vaccines

Vaccine Group	Guinea Pig No.	Units of Antitoxin per ml Diphtheria	Tetanus
I. Triple (DPT)	11	1.0	0.05
	12	4.0	0.4
	13	2.0	0.5
	14	0.5	0.8
	15	0.5	0.05
	16	4.0	0.2
	17	0.5	1.6
	18	2.0	0.8
III. Combined Triple Plus Polio (DPT-P)	61	4.0	1.0
	62	0.5	1.0
	63	2.0	0.05
	64	0.5	0.2
	65	0.5	0.25
	66	1.0	0.025
	67	1.0	0.025
	68	1.0	0.025

Note: Titrations were done in the Massachusetts Department of Health Laboratories.

jection and two weeks following each of the four injections. The serum samples were stored in the cold and tested for antibodies to each antigenic component according to the procedures described.

Results

Pertussis—The results of pertussis agglutination tests are shown in Table 4. No pertussis agglutinins were detected in any of the prevaccine specimens, nor in any bleeding from the animals injected with poliomyelitis vaccine alone. After the first injection, only one animal in each of Groups I and III had agglutinins; after the second injection, moderately high levels of agglutinins were obtained in both groups; following the third and fourth, marked increases were observed. The similarity of response in the two groups of monkeys is further illustrated graphically in Figure 3, in which the geometric mean titers are plotted. Again, as in guinea pigs tests there is every indication that the re-

Table 4—Pertussis Agglutinins in Monkeys

Vaccine Group	Monkey No.	Titers in Different Bleedings				
			Two Weeks After Each Injection			
		Prevaccine	1st	2nd	3rd	4th
I. Triple (DPT)	5,786	—	—	128	1,024	1,024
	87	—	16	256	1,024	1,024
	88	—	—	128	1,024	2,048
	GM		4	166	1,024	1,331
II. Polio	5,789	—	—	—	—	—
	90	—	—	—	—	—
	91	—	—	—	—	—
III. Combined Triple Plus Polio (DPT-P)	5,792	—	64	512	1,024	2,048
	93	—	—	128	1,024	2,048
	94	—	—	32	512	512
	GM		7	128	870	1,331

— = negative at 1:8, the lowest dilution tested.
GM = geometric mean titer.

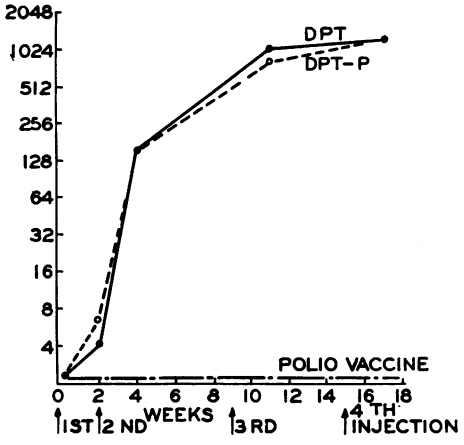

Figure 3—Geometric Mean Titers of Pertussis Agglutinins in Monkeys Bled Two Weeks Following Each of Four Injections DPT, Poliomyelitis and DPT-P Vaccines.

sponse of monkeys to the pertussis component in the combined DPT-P is as good as to the DPT alone.

Poliomyelitis—The serologic response of the monkeys to the poliomyelitis component of the vaccines is indicated by the results in Table 5. As in the case of the pertussis agglutinins no antibodies were detected in the prevaccine specimens nor did any develop in the animals receiving triple DPT alone. Of those animals receiving poliomyelitis vaccine, either alone or in combination, and subsequently tested for three types of antibodies, only three instances of antibody formation were detected two weeks after the first injection and these were Type II. The results with subsequent bleedings indicated a progressive increase of antibody of each type in all but one animal which gave practically no response to Type I. In both groups the response to Type I was uniformly lower than to the other types. In Table 6 the tabulation of geometric mean titers suggest that, in contrast to the results obtained in guinea pigs, the poliomyelitis response of the monkeys to the combined vaccine is better than to the poliomyelitis vaccine alone. This is illustrated graphically in Figure 4. However, it is pointed out that these results represent one experiment involving a small number of animals.

Diphtheria and Tetanus—The diphtheria and antitoxin titers in monkeys bled before and two weeks after each of three injections are presented in Table 7. It is readily apparent that the animals responded well to both toxoids whether in DPT or in DPT-P.

Discussion

In considering the use of combined multiple antigens, certain advantages from the administrative point of view are obvious. Fewer injections are required to accomplish the same result with less inconvenience to children and families concerned. Large numbers can be vaccinated against more diseases than might be possible if all the antigens were given separately, and protection against a disease of low incidence such as tetanus becomes practical. Synergistic effects,

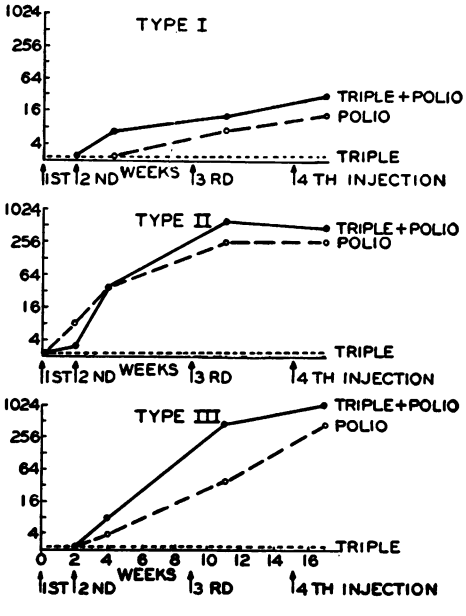

Figure 4—Geometric Mean Poliomyelitis Antibody Titers in Monkeys Two Weeks After Each of Four Vaccine Injections

Table 5—Poliomyelitis Antibodies in Monkeys

Vaccine Group	Monkey No.	Virus Type	Titers of Different Bleedings				
				Two Weeks After Each Injection			
			Prevaccine	1st	2nd	3rd	4th
I. Triple (DPT)	5,786	I	—*	—	—	—	—
	87		—	—	—	—	—
	88		—	—	—	—	—
	86	II	—	—	—	—	—
	87		—	—	—	—	—
	88		—	—	—	—	—
	86	III	—	—	—	—	—
	87		—	—	—	—	—
	88		—	—	—	—	—
II. Polio	5,789	I	—	—	—	4	—
	90		—	—	—	8	64
	91		—	—	—	8	16
	89	II	—	4	16	64	256
	90		—	—	16	1,024	256
	91		—	64	256	256	256
	89	III	—	—	—	16	64
	90		—	—	4	64	1,024
	91		—	—	8	64	1,024
III. Combined Triple Plus Polio (DPT-P)	5,792	I	—	—	64	64	256
	93		—	—	—	16	8
	94		—	—	—	—	16
	92	II	—	8	1,024	4,096	1,024
	93		—	—	16	64	256
	94		—	—	4	1,024	256
	92	III	—	—	64	4,096	1,024
	93		—	—	—	64	1,024
	94		—	—	4	256	1,024

* — = negative at lowest dilution tested (<4).

such as that of pertussis vaccine with diphtheria toxoid, may be of considerable importance.

On the other hand, there are problems involved in the production and administration of such products, as pointed out by Murray [22] and in a report of the World Health Organization.[23] If a particular combined product is to be considered acceptable, not only must there be integration as to timing of injections and dosage schedule, but the several antigens must produce at least as good response to the individual antigens as if they were used separately, and intensified local and general reactions should not follow injections. In the production of such combined antigens, certain procedures require modification and each revised method requires study before its acceptance. Furthermore, in the control of safety and potency, the number

of tests is increased since the individual components as well as the final product must be tested. However, the difficulties are not insurmountable, as demonstrated in the widely used triple DPT; and the advantages are such as to encourage experimental work to determine, if possible, the conditions under which combined antigens are applicable and to recognize possible limitations or contraindications to their use.

One question that requires further elucidation concerns a possible "crowding out" effect. In guinea pigs, Barr and Llewellyn-Jones [24] observed that antitoxic response to tetanus toxoid in mixed diphtheria-tetanus toxoids was reduced if preceded by immunization with diphtheria toxoid alone. Recently, in Formosa, Chen, et al.,[25] reported heterologous suppression of response to tetanus and pertussis antigens in children with preexisting diphtheria immunity. In the present study a possible depression of the serologic response of guinea pigs to diphtheria and tetanus toxoids and to poliomyelitis vaccine was suggested when these antigens were administered together in DPT-P. It may be pointed out, however, that the dosage schedule and intervals of bleeding may not have been optimal, and the number of animals was limited.

In contrast to the results in guinea pigs, the findings in monkeys suggest that the responses to the individual components, in general, were greater when the combined antigen was used than following DPT or poliomyelitis alone. If this truly reflects a difference in immunologic response of monkeys and guinea pigs, it illustrates the danger of generalizing on the basis of results with one species, and reminds us that conclusions about the effect of any combined antigen in human immunization ultimately must be based on immune response in human beings. In the interpretation of the reported results it is pointed out that the combined antigens were prepared in the laboratory by mixing finished vaccines—not under conditions of a manufacturing laboratory.

While experience with DPT-P in hu-

Table 6—Poliomyelitis Antibodies in Monkeys: Geometric Mean Titers

Vaccine Group	Virus Type	GM Titers at Different Bleedings				
			Two Weeks After Each Injection			
		Prevaccine	1st	2nd	3rd	4th
I. Triple (DPT)	1	—*	—	—	—	—
	II	—	—	—	—	—
	III	—	—	—	—	—
II. Polio	I	—	—	—	7	14
	II	—	8	38	256	256
	III	—	—	4	38	435
III. Combined Triple Plus Polio (DPT-P)	I	—	2	7	14	32
	II	—	—	38	666	435
	III	—	—	8	435	1,024

* — = negative in lowest dilutions tested (< 4).

Table 7—Diphtheria and Tetanus Antitoxin Titers in Monkeys Two Weeks Following Injections of Vaccine

Vaccine Group	Monkey No.	Anti-toxin	Units of Antitoxin per ml in Sera			
			Pre-vaccine	Two Weeks After Each Vaccine Injection		
				1st	2nd	3rd
I. Triple (DPT)	5,786	Diph.	< 0.001	0.001	0.05	4.0
		Tet.	< 0.001	0.001	0.05	4.0
	87	Diph.	< 0.001	> 0.005	0.5	6.0
		Tet.	< 0.001	> 0.05	0.5	> 14.0 *
	88	Diph.	< 0.001	< 0.001	0.2	4.0
		Tet.	< 0.001	0.01	0.2	1.0
III. Combined Triple Plus Polio (DPT-P)	5,792	Diph.	†	0.005	0.4	6.0
		Tet.	†	0.5	0.2	4.0
	93	Diph.	< 0.001	†	0.5	8.0
		Tet.	< 0.001	0.01 *	0.2	6.0
	94	Diph.	< 0.001	> 0.01	0.5	6.0
		Tet.	< 0.001	0.005	0.1	6.0

Note: Titrations were done in the Michigan Department of Health Laboratories.
* Insufficient for additional titrations.
† Insufficient sample.

man beings is not available it may be of interest that in a study referred to previously,[19] poliomyelitis vaccine and DPT were given to infants concurrently in different arms. Tests for poliomyelitis and pertussis antibodies in these children showed that they responded well to both antigens and there was no indication of interference.

In considering the meaning of the "crowding out" or interference effects, we should perhaps remember that the evidence presented in the literature has been based largely on primary response and, furthermore, on serologic tests. Several workers, for example di Sant' Agnese [9] and Greenberg and Fleming [26] studied antibody levels following secondary stimulus in children who had shown low levels of antibody following primary immunization in the presence of maternally transferred antibodies. These levels were comparable to those reached following a secondary stimulus in children that had shown relatively higher levels after primary immunization in the absence of maternal antibodies. The pertinent thing may well be what happens after a secondary stimulus, and in terms of actual protection against infection.

Summary

The serologic responses of guinea pigs and monkeys to triple diphtheria, pertussis and tetanus vaccine (DPT), poliomyelitis vaccine, and combined triple and poliomyelitis vaccine (DPT-P), have been studied.

All antigenic components, in whichever vaccine they were given, stimulated demonstrable antibodies.

In the present experiments with lim-

ited numbers of animals, the diphtheria and tetanus antitoxin levels in guinea pigs were somewhat lower following the combined DPT-P vaccine than following triple DPT; also, the poliomyelitis response was less following combined DPT-P than following poliomyelitis vaccine alone. In monkeys, however, all antibody levels appeared to be somewhat higher following the combined product than following poliomyelitis vaccine alone.

The pertussis serologic response in both guinea pigs and monkeys was consistently good regardless of the combination in which it was given; also, in pertussis mouse protection tests, the DPT and DPT-P vaccines were of similar potency.

The results encourage further work with combined multiple antigens in which poliomyelitis vaccine is included.

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