APPILED MICROBIOLOGY, Mar. 1968, p. 509-518
Vol. 16, No. 3
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Secondary and Apparent Primary Antibody Responses After Group A Streptococcal Vaccination of 21 Children

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Received for publication 14 December 1967

A partially purified M protein, extracted from a mouse-virulent strain of type 3, group A streptococcus, was administered subcutaneously in gradually increasing amounts at weekly intervals to 21 children in a Family Program. Seven children with type 3 bactericidal antibody in prevaccination sera showed a secondary response. Of 14 children with no detectable type 3 bactericidal antibody prior to vaccination, 13 developed definite type 3 antibody during or soon after vaccination. This response appeared to be of the primary variety in at least some of the 13 children because (i) the total incidence of antibody response (20 of 21) was greater than can be accounted for by the documented incidence of clinical and subclinical type 3 infections among children of our Family Program during a period of 14 years, (ii) the response in the 13 children with no detectable antibody prior to vaccination was more delayed than in those showing a definite secondary response, and (iii) on the average, the amount of vaccine needed for a response in these 13 children was 15 to 28 times greater than that required for the secondary response. Local skin reactions were variable among the vaccinated children. Systemic reactions were infrequent and not severe. The giving of multiple injections of partially purified M protein did not seem to cause cutaneous hypersensitivity.

There is considerable evidence that immunity to group A streptococcal infection is type-specific and is associated with the development of antibodies directed against the M antigen (7). Although animals can be successfully immunized against many different types of group A streptococci, streptococcal vaccination of man, to date, has met with only very limited success.

Among the obstacles to streptococcal vaccination of man are the existence of more than 50 different types of group A streptococci, each with its own antigen, and the fact that injection of killed streptococcal cells and streptococcal products often results in severe local or systemic reactions. In addition, since the mechanisms by which streptococcal infection bring on rheumatic fever and glomerulonephritis are not known, there is the theoretical possibility that injection of streptococcal products might cause these complications.

Attempts to prepare type-specific vaccines that would not cause severe reactions in human subjects have been made by purifying or partially purifying streptococcal M protein. Material of this kind has been used successfully to produce a secondary response (4, 10, 11). In one study (10), type-specific antibodies that had decreased to nondetectable levels were "recalled" after injection of streptococcal cell walls. In another study (4), measurable titers of hemagglutinating antibodies were increased by injection of purified soluble M protein or alum-precipitated M protein. To date, the only reported study in which a primary type-specific antibody response possibly may have been elicited in human subjects is one by Wolfe et al. (14). In this study, 10 of 16 patients given a partially purified type 14 M protein in a mineral oil emulsion developed type 14 antibodies. However, the oil emulsion caused a high incidence of local reactions, including pain, and in one instance a sterile abcess developed 2 months after the injection. Three persons also developed chills and fever.

Since primary streptococcal vaccination of human subjects would be the ideal method for preventing streptococcal infection and rheumatic fever, we have maintained an interest in this possibility for many years in spite of the known and unknown obstacles. Six years ago we embarked upon a program aimed at producing

streptococcal vaccines that could be given safely to human subjects. During the first few years, our studies were confined to animal experiments. In July 1965, we began cautiously administering to children a partially purified M protein made from a type 3, group A streptococcus that was highly virulent for mice. Our objective was to elicit a primary antibody response without causing harm, even though many injections of the vaccine might be required to do so. Although the use of multiple injections of a vaccine containing only one M antigen would not have any practical application, achieving our objective might be the first step in the development of a more practical method of immunization.

The present communication is a report of our observations on 21 children. The response of 14 children who showed no detectable type 3 bactericidal antibody in their sera prior to vaccination is compared with the response of 7 who showed either definite or borderline levels of type 3 antibody in prevaccination sera. Our observations on local and systemic reactions to the vaccine are also presented.

MATERIALS AND METHODS

Bacteria. Three mouse-virulent strains of group A streptococci, obtained from Rebecca C. Lancefield in 1961, were used in this study. The mouse virulence and M protein content of these streptococci were retained by periodic transfer through mice. One of the strains (B-930) was a type 3 organism which was used for the preparation of a partially purified M protein vaccine and for bactericidal tests to detect type 3 antibodies in human and animal sera. The other two strains were a type 1 streptococcus (T1) and a type 6 streptococcus (S43); they were used for controls whenever these were needed.

Animals. Six-week-old, white, female, Swiss mice, obtained from the Charles River Breeding Laboratories, were used for testing the toxicity and antigenic potency of vaccines made from partially purified M proteins. These mice weighed approximately 25 g each.

Bactericidal tests. The method employed is a modification of the procedure described by Lancefield (6). It depends upon the phagocytosis and destruction of streptococci by human leukocytes in the presence of M antibody. Undiluted or diluted animal and human sera were tested for type-specific antibodies by this method after being heated for 0.5 hr at 56 C and sterilized by passage through a membrane filter (Millipore Corp., Bedford, Mass.). Freshly drawn, heparinized, human blood, not containing the type-specific antibodies for which the tests were being done, served as the source of phagocytes. This donor blood was obtained from a limited number of healthy adults whose sera had been tested repeatedly for various type-specific antibodies. The group A streptococci used in the tests were obtained from a rapidly growing 3-hr culture in Todd-Hewitt broth. After adjustment of turbidity to 89 to 91% transmittance at 540 mμ in a Coleman Junior spectrophotometer, a 10^{-4} dilution of the culture was prepared and further diluted 1:4, 1:16, and 1:64 with Todd-Hewitt broth. One or more of these three culture dilutions in 0.1-ml volume was used as the inoculum. Pour plates made with 0.1 ml of the 1:4 dilution generally showed 40 to 200 colonies after overnight incubation, whereas pour plates made from the 1:64 dilution showed 2 to 15 colonies.

Tests were carried out in glass tubes (10 × 75 mm) tightly sealed with silicone-coated soft rubber stoppers. To each tube were added 0.3 ml of donor blood, 0.1 ml of serum or serum dilution to be tested, and 0.1 ml of streptococcal culture dilution. In some tests, a single culture dilution was used, but usually the tests were done in triplicate with three different dilutions. Three control tubes were included for each culture dilution used: one tube contained 0.1 ml of unabsorbed rabbit antiserum or antiserum dilution in place of the human serum; a second tube contained 0.3 ml of donor blood, 0.1 ml of Todd-Hewitt broth, and 0.1 ml of culture dilution; and a third tube contained 0.3 ml of defibrinated sheep blood, 0.1 ml of Todd-Hewitt broth, and 0.1 ml of culture dilution. All tubes were incubated at 37 C while being rotated end over end at 6 rev/min in a Scientific Industries rotator. After 3 hr, two pour plates, utilizing plain Blood Agar Base, were made from the contents of each tube. In one plate, 0.1 ml of undiluted contents was incorporated; in the other plate, 0.1 ml of a 1:100 dilution of the tube's contents was incorporated. After overnight incubation at 37 C, all of the colonies in each pour plate were counted, by use of a Darkfield Quebec Colony Counter and an electronic register. To determine the 3-hr colony count, the pour plate made from 0.1 ml of undiluted contents was used when the count was low; the pour plate made from 0.1 ml of the 1:100 dilution of contents was used when the count was high. In either case, 3-hr counts were recorded in terms of number of colonies per 0.1 ml of contents. To determine the initial or 0-hr colony count for the mixture in each tube, a fourth control tube was prepared for each of the culture dilutions used. This tube contained 0.3 ml of defibrinated sheep blood, 0.1 ml of Todd-Hewitt broth, and 0.1 ml of culture dilution. Its contents were mixed well, and then 0.4 ml was incorporated in a pour plate. After overnight incubation at 37 C, the number of colonies in the plate was counted. This number divided by 4 provided the 0-hr count per 0.1 ml.

Three conditions were required for bactericidal tests to be accepted as being valid: (i) the 3-hr colony count of the control tube containing donor blood without additional serum had to be between 500 and 30,000; (ii) the 3-hr colony count of the control tube containing rabbit antiserum had to be zero or near zero; and (iii) the 3-hr count of the tube containing defibrinated sheep blood had to be about the same or slightly less than the 3-hr count of the control tube containing donor blood without serum. The reason for the last requirement is that the type 3 streptococcal strain used, as well as a number of other strains of group A streptococci, seemed to multiply somewhat more rapidly in human blood than in defibrinated sheep

blood when type-specific antibody was absent. In addition, every unknown serum was tested in tubes containing defibrinated sheep blood plus each of the inocula in order to exclude false results due to the inadvertent presence of antibiotics.

Interpretation of tests. In the absence of type-specific antibody, streptococci containing M protein multiply rapidly in human blood during 3 hr of incubation in rotating tubes. In the presence of type-specific antibody, however, the growth is prevented or reduced. Therefore, the ratio of the 3-hr colony count for the control tube containing donor blood without additional serum to the 3-hr colony count for the tube containing donor blood plus the serum being tested can be used as an approximate measure of type-specific antibody in the serum, provided the requirements listed in the preceding paragraph are met. We have used this simple ratio as the bactericidal index of serum being tested. For the purpose of calculating this index, a 3-hr colony count of zero was recorded as 1.

On the basis of many experiments with various dilutions of type-specific rabbit antisera, we have devised the scale shown in Table 1 to convert bactericidal index of serum to antibody level. Antibody level is graded as 0, ±, +, ++, +++, or ++++. The bactericidal index used here and its interpretation are similar to, but not exactly the same as, the bactericidal index used by Stollerman et al. (12). The use of a bactericidal index facilitates interpretation of results of tests with human sera, in which the titers of type-specific bactericidal antibodies are generally much lower than in the sera of hyperimmunized animals.

In spite of the approximate relation of bactericidal index and antibody level shown in Table 1, in repeated tests with the same serum specimens we have found that results may be variable when the sera actually contain small amounts of antibody—depending on the size of the inoculum, the particular donor blood used, and other possible variables. On the other hand, when antibody is definitely absent, repeated tests consistently give bactericidal indices of less than 5, and, when appreciable amounts of antibody are present, the tests nearly always give high indices with correspondingly high (+++ or ++++) antibody levels. Because of these observations, each serum specimen was tested a number of times with inocula of various sizes and with different donor bloods. The average of the antibody levels of the different tests was used to provide a composite antibody level.

The use of the bactericidal test and its interpretation are illustrated by the data in Table 2. Results are shown for three specimens of human sera with different titers of type 3 antibody. When the sera were tested simultaneously with three different inocula of type 3 streptococci, type 3 antibody could not be detected in specimen no. 1, but it was consistently present (++++) in specimen no. 3. Tests with specimen no. 2 gave variable results (± to +++). The composite antibody level for specimen no. 2 was approximately ++. These results were confirmed by additional tests with several different donor blood specimens.

Preparation of M protein. Two kilograms (wet weight) of streptococcal cells, strain B-930 (type 3, group A), were grown for us by Difco Laboratories, Detroit, Mich. The bacteria were kept frozen at −20 C in an air-tight, sterile container, and portions were removed at the time of M protein purification. This purification was performed in two stages. The first stage was essentially the same as the method described by Lancefield and Perlmann (8). It involved heat-acid extraction of crude M protein, elimination of ribonucleic acid by digestion with ribonuclease, precipitation of M protein with ammonium sulfate, dialysis at appropriate steps in the procedure, and final drying from the frozen state. In the second stage, the resultant dry, partially purified M protein was dissolved in 0.01 M phosphate buffer to a concentration of 2.5 mg per ml and then purified further on a diethylaminoethyl (DEAE) cellulose column by the method of Kantor and Cole (5). The final product, after being frozen and dried, was a white powder.

TABLE 1. *Relation of antibody level to bactericidal index*

Bactericidal index	Antibody level
<5	0
5–24	±
25–99	+
100–299	++
300–499	+++
500 or >500	++++

This partially purified M protein (PPMP), when used to immunize mice, conferred a high degree of protection against challenge with streptococcus B-930. Actively immunized mice also developed circulating type 3 antibody that could be readily detected by the bactericidal test.

The relative M protein content of this preparation was measured by the minimal concentration which gave a definite precipitate in capillary tubes with absorbed type 3 antiserum and by comparing this concentration with that reported by others. Our material, in a number of different tests, gave a definite precipitate in concentrations of 6 to 20 μg per ml. A partially purified type 1 M protein prepared by Lancefield and Perlmann (8) gave a precipitate with absorbed homologous antiserum in concentrations as low as 5 μg per ml. A purified type 14 M protein made by Fox (2) precipitated in concentrations of 2 to 5 μg per ml.

Children selected for vaccination. Twenty-one siblings of rheumatic children participating in the House of the Good Samaritan's Family Program were selected for vaccination. This Program was described in a previous communication (9). The purpose of the vaccination trial and the precautions to be taken were explained to the children's parents, all of whom gave written permission and cooperated fully.

Prior to being given the first injection of vaccine, these children had participated in the Family Program for periods varying from 4 months to 6 years. Their streptococcal experience before, during, and after vaccination was well documented by frequent throat cultures, antistreptolysin O tests, and physical examinations—done routinely as well as at the time of all

illnesses. β-Hemolytic streptococci isolated from throat cultures were identified as far as possible by grouping and typing by the method of Swift et al. (13). Many of the serum specimens collected prior to vaccination, in addition to those collected during and after vaccination, were available for bactericidal antibody tests. One child had a documented type 3 streptococcal infection 1 year prior to vaccination. No other type 3 infections were observed prior to, during, or after vaccination.

Table 3 gives the age and sex of each of the 21 children selected for type 3 streptococcal vaccination. Data are also given in the last column of this table for the level of type 3 bactericidal antibody in serum specimens collected prior to vaccination. The sera of 7 children showed low to high levels of such antibody, whereas none of the serum specimens from the remaining 14 children showed detectable levels of type 3 antibody.

TABLE 2. *Example of bactericidal test for type 3 streptococcal antibody in three different specimens of human sera*[a]

Determination	Sheep blood control	Donor blood control	Type 3 rabbit antiserum	Serum specimens		
				No. 1	No. 2	No. 3
Zero-hour colony count, 16						
3-hr colony count	22,000	26,000	0	24,000	2,800	4
Bactericidal index			26,000	1.1	9.3	6,500
Antibody level			++++	0	±	++++
Zero-hour colony count, 6						
3-hr colony count	6,000	8,000	0	8,900	40	0
Bactericidal index			8,000	0.9	200	8,000
Antibody level			++++	0	++	++++
Zero-hour colony count, 2						
3-hour colony count	1,000	1,500	0	2,100	4	0
Bactericidal index			1,500	0.7	375	1,500
Antibody level			++++	0	+++	++++
Composite antibody level				0	++	++++

[a] All colony counts are given in terms of number of colonies per 0.1 ml of contents of tubes. Type 3 unabsorbed rabbit antiserum was used in a 1:10 dilution.

TABLE 3. *Summary of clinical material, amount of type 3 vaccine given, and amount of type 3 antibody in serum prior to vaccination*

Case no.[a]	Age (years)	Sex	Amt of M preparation given in vaccine		Composite type 3 antibody levels in sera prior to vaccination
			Total (mg)	Three largest single doses (mg)	
1	9	F	0.35	0.03 0.03 0.02	± to +
2	9	F	0.68	0.15 0.10 0.10	None
3	7	M	1.08	0.15 0.15 0.15	± to +
4	12	M	1.49	0.15 0.15 0.15	None
5	7	F	1.77	0.40 0.20 0.20	None
6	14	M	1.88	0.20 0.20 0.15	+ to ++
7	9	F	1.89	0.40 0.20 0.20	+++ to ++++
8	8	M	1.99	0.40 0.20 0.20	± to +
9	14	M	2.15	0.30 0.20 0.20	None
10	12	F	2.24	0.50 0.25 0.20	None
11	10	F	2.27	0.50 0.25 0.20	None
12	9	F	2.41	0.25 0.20 0.20	None
13	8	F	2.49	0.60 0.30 0.25	None
14	10	F	3.35	0.80 0.40 0.35	± to +
15	14	M	3.58	0.40 0.35 0.35	None
16	9	M	4.38	1.00 0.50 0.45	None
17	9	M	4.57	1.00 0.50 0.45	None
18	9	M	4.72	0.80 0.40 0.40	+++ to ++++
19	9	M	5.72	1.00 0.60 0.55	None
20	8	M	5.72	1.00 0.60 0.55	None
21	12	M	6.07	1.00 0.65 0.60	None

[a] Cases are listed in order of total amount of vaccine given.

Preparation and administration of type 3 streptococcal vaccine. A stock solution of type 3 vaccine was prepared by dissolving the dry PPMP in sterile normal saline with 1:10,000 Merthiolate at a concentration of 1.0 mg per ml. After passage of the stock solution through a Millipore filter, dilutions from 1:2 to 1:10,000 were also made, under sterile precautions, in normal saline with 1:10,000 Merthiolate.

Before being administered to human subjects, the stock solution of vaccine and all dilutions of it were tested several times for sterility on blood-agar plates and in a liquid medium containing thioglycolate. In addition, the PPMP was administered by intraperitoneal and subcutaneous routes to mice without any apparent untoward effect. The mice, which were observed for 7 to 21 days after all injections, were given various amounts of PPMP up to a maximum of 1.0 mg (dry weight) per injection, for a total of three to five injections on alternate days. On the basis of body weight, the 1.0 mg dose was approximately 800 to 36,000 times the maximal single doses that were given later to children.

Initial tests with dilutions of the stock vaccine were done on 4 adult volunteers. Subsequently, a total of 18 to 33 injections of vaccine were given at about 1-week intervals to the 21 children selected from our Family Program. The first injection consisted of 0.1 ml of the 1:10,000 dilution given intradermally. The intradermal dose was gradually increased to 0.1 ml of the 1:100 dilution. When it became apparent that this latter amount caused either no reaction or at the most a very small area of erythema, 0.1 ml of the 1:100 dilution was given subcutaneously. This same dose was repeated several times, and then, between 6 and 9 September 1965, 0.2 ml of the 1:100 dilution (0.002 mg of PPMP) was given to all 21 children. Thereafter, the amount administered was cautiously increased according to the tolerance of each child. Whenever an injection of vaccine was followed by an appreciable local reaction or a possible systemic reaction, either the same amount or a smaller amount was given at the next weekly injection. If this injection caused no reaction or only a mild local reaction, the dose was again increased at subsequent injections.

In this way, severe reactions were avoided, and it was possible to increase the amount of vaccine administered to the levels shown in Table 3. Data in Table 3 provide information as to the cumulative total amount of vaccine and the amounts in the three largest single doses administered to each of the 21 children; all amounts are given in terms of milligrams (dry weight) of PPMP. Not shown in the table is the fact that, on the basis of body weight, the maximal single dose varied from 0.001 to 0.047 mg per kg.

RESULTS

Bactericidal antibody response in children not showing type 3 antibody prior to vaccination. Type 3 bactericidal antibodies could not be detected in serum specimens collected from 14 children prior to vaccination. One child (case 2), who was given relatively small amounts of vaccine, failed to develop type 3 antibody. All of the remaining 13 children, sooner or later, developed high levels of type 3 bactericidal antibody.

Table 4 summarizes the results of tests with serum specimens collected from all 13 children who showed a definite bactericidal antibody

TABLE 4. *Composite type 3 antibody levels of serum specimens from 13 children not showing type 3 antibody prior to vaccination*

Case no.[a]	Relative time of serum collection								
	Prior to any vaccine	Between initial injection of vaccine and first injection of 0.04 mg of M preparation	Months following injection of vaccine[b] containing 0.04 mg of M preparation						
			<1	1-2	2-3	3-4	4-5	5-6	6-7	7-8
4	0 0 0 0	0 0 0 0	0	0	±*		+		++++	++++
5	0	0 0 0	0	0	+*	+++	+++	++++	++++	
9	0 0 0 0 0 0	0 0 0 0	±		+	+++*	++++	+++	++++	++++
10	0 0 0	0 0 0	0	+	+*	+++		++++	++++	++++
11	0	0 0 0 0	0	±	++*	++++	++++	++++	++++	++++
12	0 0 0 0 0 0	0 0 0	0	+	++*	+++	++++	++++	++++	++++
13	0 0 0 0	0 0 0 0	±	++	++*	+++	+++	++++	++++	
15	0 0 0 0 0 0	0 0	0	0	0	0	±	+++*	++++	++++
16	0 0 0 0 0 0	0 0	0	0	±	++*	+++	+++	++++	++++
17	0 0 0 0	0 0	0	0	0	0*	±	++	+++	++++
19	0 0 0 0 0 0	0 0 0	±	+	+	++*	++++	+++	++++	++++
20	0 0 0	0 0 0	0	±	+++	++++*	++++	+++	++++	++++
21	0 0 0 0	0 0	0	±	±	±*	±	++	++++	++++

[a] Case 2, who was given only small amounts of vaccine and who did not develop detectable antibody, is not included.

[b] Asterisk indicates approximately when the last injection of vaccine was given.

response to type 3 vaccine. Results are given as composite antibody levels. The table is constructed so as to show composite antibody levels for serum specimens collected prior to the administration of any vaccine, during the administration of relatively small amounts of vaccine (amounts containing less than 0.04 mg of PPMP), during the administration of larger amounts of vaccine, and after the discontinuation of vaccination.

From the data in Table 4, it is evident that type 3 antibody could not be detected in any of the serum specimens collected prior to the administration of type 3 vaccine nor in any of the specimens collected prior to the injection of amounts of vaccine containing 0.04 mg of PPMP. Subsequent to the injection of this or greater amounts of vaccine, type 3 bactericidal antibody began to appear and finally reached high composite levels (++++) for all of the 13 children.

Injections were discontinued about the same time (31 January to 2 February 1966) in 12 of the 13 children. In case 15, however, the period of vaccination was extended to 30 March 1966 because preliminary tests of serum specimens collected through the end of January 1966 showed that type 3 antibody had not yet appeared. By 31 January 1966, type 3 antibody had also failed to appear in serum specimens collected from case 17. Although vaccination was not continued beyond this date, type 3 bactericidal antibody was detected in the serum specimen collected on 28 February and subsequently rose to a high level (+++ to ++++).

Bactericidal antibody response in children showing type 3 antibody in sera collected prior to vaccination. Serum specimens collected prior to the administration of type 3 vaccine showed varying levels of type 3 bactericidal antibody in seven children. One of these was the child who had a documented type 3 streptococcal infection 1 year prior to vaccination. In all of these seven children, the titer of type 3 antibody increased during the course of vaccination.

Data showing these secondary antibody responses to type 3 vaccine are summarized in Table 5. Here, maximal composite type 3 antibody levels are given for undiluted sera and for various dilutions of sera collected prior to the first injection of vaccine. For comparison, similar data are given for those serum specimens that showed the highest titer of type 3 antibody after vaccination. Prior to vaccination, the maximal composite antibody level of undiluted serum was + for four children (cases 1, 3, 8, and 14), ++ for one child (case 6), and ++++ for two children (cases 7 and 18). For those children with only + or ++ composite antibody levels in undiluted serum, bactericidal activity was lost when the sera were diluted 1:5 or 1:10. For those with ++++ composite antibody levels in undiluted serum, bactericidal activity was not lost until the sera were diluted 1:30 (case 18) or 1:60 (case 7). After vaccination, maximal composite antibody levels of undiluted serum were ++++ for all seven children, and bactericidal activity was retained in higher serum dilutions than it was in dilutions of sera collected prior to vaccination.

By comparing the composite antibody levels for various dilutions of prevaccination sera with those of postvaccination sera, it is possible to estimate the increase in type 3 antibody titer that

TABLE 5. *Maximal composite type 3 antibody levels of various dilutions of sera collected prior to vaccination and after vaccination of seven children showing a secondary response*[a]

Dilution of serum	Case 1		Case 3		Case 6		Case 7		Case 8		Case 14		Case 18	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Undiluted	+	++++	+	++++	++	++++	++++	++++	+	++++	+	++++	++++	++++
1:2					+	+++								
1:3					+	++++			±	++++				
1:5	0	++++	±	++++	+	+++			0	+++	0	++++		
1:10	0	++++	0	+++	0	++	++	++++	0	+	0	++++	++++	++++
1:15			0	++									++++	++++
1:20	0	++	0	+	0	+	+	++++					+	++++
1:30							±	++++			0	+++	0	++++
1:40	0	++	0	0			±	++++	0	±				
1:50			0	0			±	+++			0	++	0	++++
1:60	0	+					0	++					0	++++
1:80							0	+			0	±	0	+++
1:100							0	±					0	+
1:500													0	0

[a] Pre indicates serum specimens that were collected prior to vaccination. Post indicates serum specimens that were collected after administration of type 3 vaccine.

resulted from the administration of type 3 vaccine. This increase of titer varied from as little as 2- to 6-fold (case 7) to as much as 60-fold (case 1).

Comparison of antibody response of children showing type 3 antibody initially with response of children showing no type 3 antibody prior to vaccination. The antibody responses observed in 20 of the 21 vaccinated children have been classified tentatively in Table 6 as "secondary" or "primary," according to whether or not type 3 bactericidal antibody could be detected in serum specimens collected prior to the first injection of type 3 vaccine. Data in this table show the time required for an antibody response, as well as the maximal amount of PPMP given in a single injection and the cumulative amount of PPMP given up to 7 to 14 days prior to detection of an antibody response.

TABLE 6. *Comparison of "secondary" and "primary" antibody response to type 3 vaccine with regard to time required for response and amount of vaccine given*

Tentative classification of antibody response	Case no.[a]	Time required for antibody response[b] (weeks)	Maximal amount of M preparation given 7 to 14 days prior to rise in titer or first appearance of antibody	
			In single injection (mg)	Cumulative total (mg)
Secondary	1	2	0.002	0.01
	3	5	0.02	0.04
	6	3	0.01	0.03
	7	6	0.02	0.07
	8	5	0.02	0.04
	14	1	0.002	0.009
	18	7	0.04	0.12
	Avg	4	0.016	0.046
Primary	4	19	0.15	0.10
	5	19	0.20	1.08
	9	7	0.04	0.13
	10	15	0.15	0.69
	11	13	0.10	0.43
	12	15	0.20	0.90
	13	11	0.05	0.20
	15	24	0.40	3.28
	16	15	0.20	1.12
	17	25	1.00	4.56
	19	13	0.30	1.21
	20	13	0.30	1.21
	21	9	0.10	0.31
	Avg	15	0.245	1.248

[a] Case 2, the only child who showed no antibody response, is not included in this table.

[b] Time required for an antibody response is recorded as number of weeks intervening between first injection of 0.002 mg of PPMP and first rise in titer or first detection of type 3 bactericidal antibody.

The time required for an antibody response is recorded as the number of weeks intervening between first injection of 0.002 mg of PPMP and first detection of the antibody response. Time of injection of 0.002 mg of PPMP was selected as the reference point because all 21 children were first given this amount at about the same time (7 September to 9 September 1965) and because only very small amounts of vaccine had been given prior to that. The time required for an antibody response was 7 weeks for one child with a "secondary" response (case 18) and one child with a "primary" response (case 9). For the remaining 6 children showing a "secondary" response, the time was less than 7 weeks, and for the remaining 12 children with a "primary" response the time was greater than 7 weeks. For 7 of the 13 children with a "primary" response, the time was 15 to 25 weeks.

The data in Table 6 show that nearly all "secondary" responses occurred not only earlier than "primary" responses but that they also occurred after the administration of smaller amounts of vaccine. Thus, in 6 of 7 children with a "secondary" response, the maximal amount of vaccine given in a single injection prior to this response contained 0.02 mg of PPMP or less, whereas in 12 of 13 children classified as having a "primary" response the corresponding dose was 0.05 mg or more. The two groups showed a slight overlap in that one of the children with a "secondary" response (case 18) and one with a "primary" response (case 9) received the same amount of vaccine (0.04 mg PPMP) within 7 to 14 days before the antibody response was noted. These were the same two children for whom the time required for a response was the same. Furthermore, the cumulative amounts of vaccine given to these two children prior to detection of their antibody responses were very similar.

The average amounts of vaccine given to the 7 children with a "secondary" response and the 13 children with a "primary" response are also shown in Table 6. It is apparent that the averages are considerably greater for those with a "primary" response than for those with a "secondary" response. The ratio of these averages is 15:1 for the maximal amount given in a single injection and 28:1 for the cumulative total amount of vaccine given.

Type specificity of the antibody response to type 3 vaccine. To determine whether the response to type 3 vaccine was type-specific, bactericidal tests were done for the presence of type 1 and type 6 antibodies, as well as for type 3 antibodies, in a large number of serum specimens collected be-

fore, during, and after the administration of type 3 vaccine. In serum specimens collected prior to the administration of type 3 vaccine, variable levels of bactericidal antibody were present for type 3 in 7 children, for type 1 in 8 children, and for type 6 in 7 children. In no instance was the administration of type 3 vaccine accompanied by the appearance of type 1 or type 6 antibodies when these antibodies were absent in prevaccination sera nor by an increase in levels of type 1 or type 6 antibodies when these antibodies could be detected in the prevaccination sera.

Reaction to intradermal vaccine. All of the 21 children were given 0.1 ml of the 1:100 dilution of the stock vaccine solution intradermally prior to subcutaneous vaccination, and all were retested with this same amount intradermally about 1 week after completion of their course of vaccination. In 9 children, the initial skin test resulted in an area of erythema between 0.5 and 3.0 cm in diameter. In three of these children, the area of erythema was accompanied by slight induration. This local reaction reached its maximal intensity about 24 hr after intradermal injection. The postvaccination intradermal skin tests resulted in a similar degree of reaction in 7 of the 21 children. Only three of the children with positive prevaccination tests were among those showing a positive postvaccination test. Six children with an initial positive test showed a negative reaction after vaccination, and four with a negative initial test had a positive postvaccination test. In eight children, the intradermal test was negative both before and after the course of subcutaneous vaccination.

Reactions to subcutaneous vaccine. The initial subcutaneous dose of vaccine was 0.1 ml of a 1:100 dilution of the stock solution (0.001 mg of PPMP). This amount, which was the same as that used for the intradermal skin tests, failed to cause a local or systemic reaction in any of the 21 children. Subsequently, the amount of vaccine administered subcutaneously at about 1-week intervals was increased very gradually until the severity of reactions made it seem inadvisable to increase the dose further or until the dose reached 1.0 ml of the undiluted stock vaccine solution (1.0 mg of PPMP).

In general, the maximal amount of vaccine given in a single injection, shown in Table 3, was determined by the development of local reactions. For 20 of the 21 children, the maximal dose was between 0.15 and 1.0 mg of PPMP. In one child (case 1), the maximal tolerated dose was only 0.03 mg. Local reactions usually consisted of areas of erythema, varying in size from 2.0 to 10.0 cm in diameter, within which were smaller areas of slight swelling and slight-to-moderate tenderness. In all instances, they subsided within 1 to 3 days. In many instances, an amount of vaccine that gave a slight-to-moderate reaction could be given later without causing any reaction.

The 21 children were given a total of 603 injections of PPMP in varying amounts. Twelve of these injections, in 10 children, possibly caused some systemic reaction in addition to an accompanying local reaction. In eight instances fever was present. One child developed a maximal oral temperature of 103 F. However, this fever could have been caused by an upper respiratory infection that began on the same day. In the other seven children with fever, maximal oral temperatures were 99.6 to 101 F; one of these seven children also had an upper respiratory infection. There were four instances of mild headache or slight-to-moderate malaise without fever which may have been due to a systemic reaction to the streptococcal vaccine.

Rheumatic fever. One of the vaccinated children developed definite rheumatic fever and a second child developed an illness with joint pain. These two cases will be described in a separate report (*manuscript in preparation*).

DISCUSSION

In this study, the bactericidal test was selected as the method for determining whether an antibody response was elicited by the type 3, group A streptococcal vaccine administered to 21 children because the bactericidal reaction is considered to be the most reliable test for determining type-specific streptococcal antibodies in human sera (7) and because bactericidal antibody has been shown to correlate well with protective antibody (6).

The technique of measuring type-specific antibodies to group A streptococci by hemagglutination (HA) of tanned red blood cells sensitized with M protein (1, 2) appears to be more sensitive than the bactericidal test. However, the HA test is so sensitive that hemagglutination with serum concentrations greater than 1:100 is not significant (2). Although several rabbit sera with HA titers of 3,200 to 204,800 were shown to have bactericidal antibodies and to confer passive protection to mice against challenge with lethal doses of homologous group A streptococci (2, 3), correlation in human subjects of protective antibody or immunity with HA antibody has not yet been clearly demonstrated. In tests with human sera, Fox et al. (4) observed bactericidal activity in 10 specimens with HA titers of 1,600 to 102,400, but failed to detect bactericidal activity in 5 specimens with HA titers of 800 to 12,800.

The data collected in the present study demon-

strate that all but 1 of 21 children given type 3 streptococcal vaccine showed a type-specific bactericidal antibody response. Seven children who had varying levels of type 3 antibody in serum specimens collected prior to vaccination developed higher titers of type 3 antibody during and following vaccination. In these children, the response obviously was of the secondary variety.

With regard to the development of type 3 bactericidal antibody by 13 of the 14 remaining children who failed to show such antibody in their sera prior to vaccination, the question arises as to whether the response was primary and whether, therefore, the objective of our study was fulfilled. As other investigators (10) have pointed out, the apparent absence of antibody prior to vaccination does not in itself prove a primary response, since our 13 children conceivably could have had type 3 streptococcal infection some time in the past and antibody titers following infection may have fallen subsequently to such low levels that they could not be detected by the bactericidal test. On the other hand, the available evidence suggests that the observed response was primary in *at least some* of these 13 children. This evidence is as follows.

If the response in the 13 children without detectable type 3 antibody prior to vaccination was secondary in all instances, it would follow that all responses observed in 20 of 21 vaccinated children were secondary and that all of these 20 children had had previous clinical or subclinical type 3, group A streptococcal infection. The 21 vaccinated children lived for a total of 208 person-years from birth to date of first vaccination. To account for 20 secondary responses, it would be necessary for the 21 children to have experienced type 3 infections at an average rate of 0.096 per person year. Since this rate is more than three times the actual average rate (0.03) of type 3 infection per person-year observed during the 14 years that our Family Program has been conducted, the conclusion that all 20 antibody responses were secondary does not seem likely.

In comparison to the definitely secondary responses in seven children, the antibody responses were more delayed and were not detectable until after the administration of larger single doses and larger cumulative amounts of vaccine in all but 1 (case 9) of the 13 children who showed no type 3 antibody in prevaccination sera. On the average, the maximal amount of type 3 M preparation given 7 to 14 days before a detectable antibody response was 15 to 28 times greater for these 13 children than for the 7 children with a recognized secondary response. These differences, shown in Table 6, are consistent with the well known behavior of secondary and primary antibody responses; hence, they support the view that the responses were primary in *at least some* of the 13 children.

The maximal single injection of PPMP that was given 7 to 14 days before detection of a secondary response in seven children varied from 0.002 to 0.04 mg (Table 6) and averaged 0.016 mg. In five of the seven children, the amount was between 0.01 and 0.04 mg. It is of interest that these amounts do not differ appreciably from the 0.02-mg dose of a purified type 24 M protein observed by Fox et al. (4) to give a secondary antibody response in 11 adults. However, differences in method of purification of M protein and in method of administration of vaccine do not allow for an accurate comparison of our data with the data of these other investigators.

Our data suggest that the amount of M protein required to elicit a primary antibody response may average 15 to 28 times more than the amount needed to produce a secondary response. This observation, if confirmed, may pose a difficult problem in the development of a practical method of vaccination. On the other hand, among the 13 children showing an apparent primary response, the maximal dose of PPMP given in a single injection 7 to 14 days before detection of type 3 antibody (Table 6) was less than the maximal tolerated dose in 10 of these children and the same as the maximal tolerated dose given to 3 children (Table 3). Therefore, the problem of developing a practical vaccine may not be insurmountable, especially if efforts, such as those reported by Fox et al. (4), are successful in reducing vaccine requirements by the use of highly purified M protein preparations and the use of adjuvants.

There is no evidence from our data that multiple injections of the partially purified type 3 M protein caused cutaneous hypersensitivity. Local reactions to subcutaneous injections of vaccine varied among the 21 children, but in no instance was the reaction severe. In general, the frequency of reactions and the size of areas of erythema in each child were related to dose.

With regard to possible reactions to the type 3, group A vaccine, the most important consideration is whether vaccination could have contributed in any way to the development of definite rheumatic fever in one child and to an illness with joint pain in a second child. This problem will be discussed in a separate report (*manuscript in preparation*).

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant HE-04957 and contract PH-108-64-55, and by grants from the Plymouth County and Greater Boston chapters of the Massachusetts Heart Association.

We are grateful to Trudy Wagner and Robert Perkins for able technical assistance.

LITERATURE CITED

1. DENNY, F. W., JR., AND L. THOMAS. 1953. The demonstration of type-specific streptococcal antibody by a hemagglutination technique employing tannic acid. J. Clin. Invest. **32**:1085–1093.
2. FOX, E. N. 1964. Antigenicity of the M proteins of group A hemolytic streptococci. J. Immunol. **93**:826–837.
3. FOX, E. N., AND M. K. WITTNER. 1966. Antigenicity of the M proteins of group A hemolytic streptococci. II. Antibody response in rabbits to vaccines prepared with oil emulsions and aluminum hydroxide. J. Immunol. **97**:86–94.
4. FOX, E. N., M. K. WITTNER, AND A. DORFMAN. 1966. Antigenicity of the M proteins of group A hemolytic streptococci. III. Antibody responses and cutaneous hypersensitivity in humans. J. Exptl. Med. **124**:1135–1151.
5. KANTOR, F. S., AND R. M. COLE. 1960. Preparation and antigenicity of M protein released from group A, type 1 streptococcal cell walls by phage-associated lysin. J. Exptl. Med. **112**: 77–96.
6. LANCEFIELD, R. C. 1957. Differentiation of group A streptococci with a common R antigen into three serological types, with special reference to the bactericidal test. J. Exptl. Med. **106**:525–544.
7. LANCEFIELD, R. C. 1962. Current knowledge of type-specific M antigens of group A streptococci. J. Immunol. **89**:307–313.
8. LANCEFIELD, R.C., AND G. E. PERLMANN. 1952. Preparation and properties of type-specific M antigen isolated from a group A, type 1 hemolytic streptococcus. J. Exptl. Med. **96**:71–97.
9. MILLER, J. M., S. L. STANCER, AND B. F. MASSELL. 1958. A controlled study of beta hemolytic streptococcal infection in rheumatic families. I. Streptococcal disease among healthy siblings. Am. J. Med. **25**:825–844.
10. POTTER, E. V., G. H. STOLLERMAN, AND A. C. SIEGEL. 1962. Recall of type-specific antibodies in man by injection of streptococcal cell walls. J. Clin. Invest. **41**:301–310.
11. SCHMIDT, W. C. 1960. Type-specific antibody formation in man following injection of streptococcal M protein. J. Infect. Diseases **106**:250–255.
12. STOLLERMAN, G. H., F. S. KANTOR, AND B. D. GORDON. 1958. Accessory plasma factors involved in the bactericidal test for type-specific antibody to group A streptococci. I. Atypical behavior of some human and rabbit bloods. J. Exptl. Med. **108**:475–491.
13. SWIFT, H. F., A. T. WILSON, AND R. C. LANCEFIELD. 1943. Typing group A hemolytic streptococci by M precipitin reactions in capillary pipettes. J. Exptl. Med., **78**:127–133.
14. WOLFE, C. K., JR., J. A. HAYASHI, G. WALSH, AND S. S. BARKULIS. 1963. Type-specific antibody response in man to injections of cell walls and M proteins from group A type 14 streptococci. J. Lab. Clin. Med. **61**:459–468.