ANTIGENICITY OF THE M PROTEINS OF GROUP A HEMOLYTIC STREPTOCOCCI

III. Antibody Responses and Cutaneous Hypersensitivity in Humans*

By EUGENE N. FOX, Ph.D., M. K. WITTNER, and ALBERT DORFMAN, M.D.

(From the La Rabida-University of Chicago Insitute, Department of Microbiology and Department of Pediatrics, University of Chicago, Chicago)

(Received for publication 22 August 1966)

Epidemiological evidence and laboratory experimentation over the past 20 yr supports the conclusion that immunity to group A streptococcal infection is type-specific. Antibodies directed against the M proteins, the antigens responsible for the specificity of the 50 or more serotypes of group A streptococci, are protective by virtue of their opsonic capacity. This subject has been thoroughly reviewed by Lancefield (1). Recent work has demonstrated that several small doses, e. g. 10 μg, of highly purified M proteins induce type-specific bactericidal antibodies in rabbits (2). The success of these latter experiments has been the impetus for this investigation on the development of a streptococcal vaccine for human use. With highly purified M proteins of types 12, 14, and 24, we have examined the degree of cutaneous hypersensitivity and the level of circulating antibodies in adults and infants. These studies have furnished data on the relative tolerance to M proteins and the possible extent of previous exposure to these serotypes. Secondary bactericidal antibody responses have been induced in adults injected with M protein vaccines without provoking untoward local or systemic reactions. It is the purpose of this report to examine the feasibility of type-specific immunization of humans against group A streptococcal infections.

Materials and Methods

M Protein.—Highly purified M proteins were prepared from Group A streptococcal cell walls as previously described (3). A slight modification of the chromatographic procedure eliminated traces of nonspecific antigens. To a column of carboxymethyl cellulose equilibrated with 0.03 M sodium acetate and containing the M protein sample was added 0.1 M sodium acetate buffer at pH 5.5. When the effluent pH reached 5.5, a linear gradient of 0.1 M potassium phosphate buffer of increasing pH was applied to the column by adding equal volumes of buffers at pH 6.0, 6.5, and 7.0 to a Technicon "Autograd" mixing chamber for the final elution. Minor fractions of nonspecific protein emerged prior and subsequent to the elution of the main peak of M protein near pH 6.

* Supported by grants from the United States Public Health Service (AM-05996 and AI-04342).

All buffers used in the chromatographic procedures and for antigen dilutions were prepared with pyrogen-free sterile water. Ringer's lactate buffer (Baxter Laboratories, Inc., Morton Grove, Illinois) was the final diluent for all parenteral M protein preparations which were sterilized through Millipore filters before dilution. Standard procedures for sterility, pyrogenicity, and toxicity testing were followed as prescribed by the United States Public Health Service (4); all M protein preparations met the requirements for these tests. Alum-precipitated M protein (APM) was prepared and standardized according to methods described previously (2).

Serological Procedures.—Type-specific precipitin reactions were carried out in capillary tubes according to Swift et al. (5), with specific rabbit antisera obtained from the United States Communicable Disease Center, Atlanta, Georgia. Antisera for hemagglutination standards were from rabbits immunized with purified M proteins in incomplete Freund's adjuvant (2). The hemagglutination (HA) assay of type-specific antisera was performed according to our standard technique (6). Sera from adults were obtained from clotted blood obtained by venipuncture. Peripheral blood of infants was obtained by heel puncture. The initial dilution for all HA assays was 1:100; sera not reacting at this concentration were considered negative. The geometric mean HA titers were calculated as antilog $\left(\frac{1}{n}\sum \log A_n\right)$ where A_n equal the reciprocals of the highest positive dilutions of n samples.

The indirect bactericidal test was performed essentially as described by Lancefield (7) with slight modification (2). A bactericidal index (8) scale from zero (no inhibition) to 4 (complete phagocytosis) has been devised. This scale is based on the ratio R of the total colony count of the inoculum at dilutions of 1:16, 1:64, and 1:256 to the total number of colonies at these dilutions in the presence of antibody. The 5 values for R chosen on the basis of accumulated data, are grouped as 10 or greater, 9 to 5, 4 to 2, 1, and less than 1, and these groups correspond to index values of 4, 3, 2, 1, and 0, respectively. R is significant only when the total colonies obtained after 3 hr in the presence of normal rabbit serum minus the original inoculum is greater than 1500. For example, a serum allowing a total surviving colony count of 43, when the initial inoculum was 215 would have a value for R of 215/43 or 5, and thus a bactericidal index of 3. This method of computation of the bactericidal index is simple and permits an index for sera that completely eliminate all streptococci in the phagocytic system.

Clinical Procedures.—Cutaneous hypersensitivity was determined by injecting intracutaneously on the volar surface of the forearm 0.1 ml of sterile M protein in Ringer's lactate buffer. Subcutaneous injections of soluble M protein or AMP in 0.5 ml of Ringer's lactate buffer were administered in the deltoid region. Subjects with histories or symptoms of heart or kidney disease or chronic streptococcal infections were excluded from the study.

Gel Electrophoresis.—Acrylamide gel disc electrophoresis was performed with a Model 6 Canalco apparatus by methods described (3). For the isolation of the multiple fractions comprising the M protein, a preparative gel electrophoresis column 1.8 × 8 cm was constructed similar to that described by Lewis and Clark (9). 10 mg samples of M protein were separated into constituent fractions under a potential of 400 v at 35 ma for 90 min at room temperature. Small-bore longitudinal cores were cut from the large columns of gel and stained. Slices corresponding to areas of stained protein were cut from the main columns of gel, and protein was recovered from the slices by electrophoresis in dialysis sacks. The eluted protein was concentrated by lyophilization, dialyzed against Ringer's lactate buffer at 5°C, and sterilized by filtration through Millipore membranes. For immunodiffusion reactions it was discovered that columns of gel with stained bands of M protein could be soaked in buffered saline for several hours to remove the acetic acid and the stained M protein bands were serologically active when sections were embedded in agar.

RESULTS

Cutaneous Hypersensitivity and Circulating Antibodies to M Proteins.—Adult volunteers between 21 and 35 yr of age, selected from a population of medical students and laboratory personnel, were skin-tested with 1 μg intracutaneous doses of purified M proteins. Concurrently, serum antibody titers were assayed by passive hemagglutination (HA). Table I summarizes the data accumulated in 106 tests in 91 persons of whom 15 were tested with both M protein serotypes. In the two groups of subjects, 70 and 84% exhibited a delayed cutaneous hypersensitivity (DCH) to M serotypes 12 and 24, respectively, and over 90% of both groups had circulating antibodies with titers of 100 or greater. DCH was considered positive when areas of erythema were greater than 0.5 cm in diameter at the height of the reaction. Extremely reactive subjects had erythematous reactions up to 6 cm accompanied by swelling and induration. HA titers in a few individuals increased two- to fourfold 1 wk after the skin testing; the values used to calculate the geometric mean titers were those obtained from sera taken immediately prior to the intracutaneous injection. The geometric mean titer following the skin tests increased slightly, but was not considered significant in this group. It is noteworthy that there was no correlation between an increase in titers and degree of DCH, nor was there a correspondence of either reaction with age, sex, or race. The absence of DCH was not correlated with a lack of circulating antibodies. In summary, a large majority of the normal young adults tested had circulating antibodies and exhibited a DCH to types 12 and 24 M proteins.

TABLE I

Hemagglutination Titers and Delayed Hypersensitivity to Types 12 and 24 M Proteins in Adults

Distribution	Type 12	Type 24
Number of subjects tested*	68	38
Positive delayed cutaneous hypersensitivity (DCH)	48 (70%)	32 (84%)
Hemagglutination (HA) titer > 100	64 (94%)	31‡ (97%)
Geometric mean titer	1840	760
Positive DCH and positive HA	46 (68%)	27 (84%)
Positive DCH and negative HA	3 (4%)	0
Negative DCH and positive HA	18 (26%)	4 (12%)
Negative DCH and negative HA	1	1

* 15 subjects were tested for both serotypes.

‡ Sera of 6 subjects were unavailable for HA titration.

In contrast to adults, a significantly lower order or reactivity was observed in a group of 59 infants between the ages of 6 months and 3 yr (Table II). These children were tested with types 14 and 24 M proteins in the same manner as the adult group. It may be seen that 8% of the skin tests and 13% of the HA assays (of sera taken subsequent to skin testing) were positive. 42 of the 59 infants had neither circulating antibodies nor exhibited a DCH to either M protein; this may be compared to 2% of the adults who were negative in both

TABLE II

Hemagglutination Titers and Delayed hypersensitivity to Types 14 and 24 M Proteins in Infants

Distribution among 59 subjects, ages 6 months to 3 yr*	Type 14	Type 24
Positive delayed cutaneous hypersensitivity‡ (DCH)	4	5
	8%	
Hemagglutination (HA) titers greater than 100 before skin test	1	2
HA titers greater than 100 7 days after skin test§	3	11
	13%	
Positive DCH and positive HA	1‖	3
Positive DCH and negative HA	2	1
Negative DCH and positive HA	2	8
Negative DCH and negative HA	42	43
	77%	

* 59 infants tested with 2 serotypes; 8 sera were unavailable for type 14 HA tests 7 days after inoculation.

‡ 2 subjects exhibited DCH to both serotypes.

§ Geometric mean titer of 1000 for both serotypes calculated together.

‖ This subject exhibited DCH and positive HA titers for both serotypes.

respects. In the infants as in the adults there appeared to be no correlation between skin reactivity and circulating type-specific antibody, nor was there a correlation of these parameters with sex or race. 25 of the infants in this group were under 1 yr of age; none had circulating antibodies or DCH to the M proteins. The increase in HA titers of 8 subjects subsequent to skin-testing may indicate that a 1 μg dose of M protein induces some degree of active immunization.

Nature of the Delayed Cutaneous Response.—All putatively hypersensitive persons reacted to intracutaneous injections of purified M proteins in the same general manner. Erythema appeared in about 6 hr, reached a maximum between 18 and 24 hr, and virtually disappeared within 72 hr; the more intense reactions

were accompanied by swelling and induration. The lack of reactivity in approximately 20% of the adults and 90% of the infants and the differences in the reactions to several serotypes among individuals indicated that the cutaneous reactions were in all likelihood a manifestation of delayed hypersensitivity to M proteins and not a result of nonspecific toxicity to undetected streptococcal antigens.

In order to demonstrate further that type 12 M protein per se was responsible

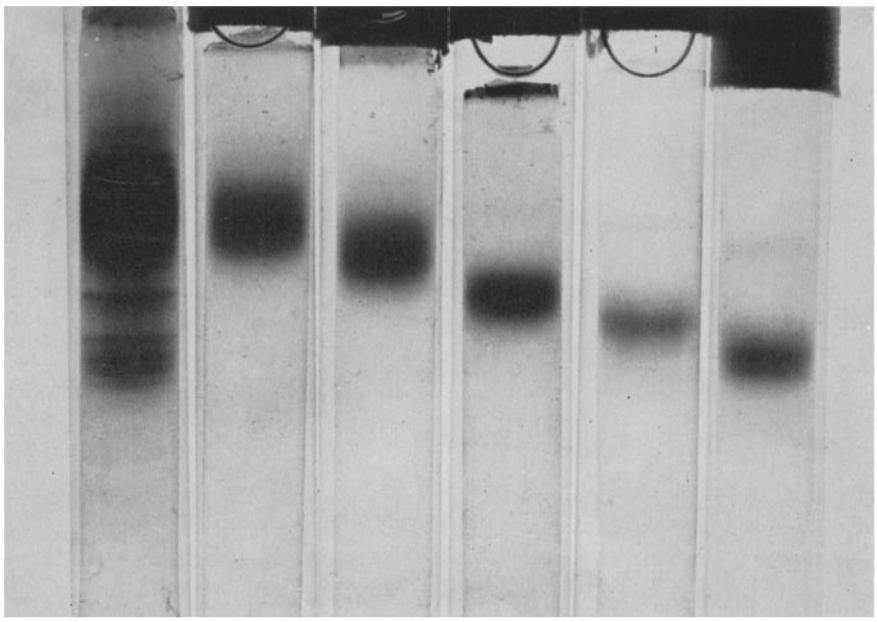


FIG. 1. Acrylamide gel electrophoretic patterns of type 12 M protein, showing the multiple molecular structure (left) and 5 fractions obtained from a preparative electrophoretic gel column.

for the DCH, the M protein was fractionated by acrylamide gel electrophoresis into its multiple components (3). We had previously shown by gel electrophoresis that purified M proteins extracted from cell walls by hot acid are obtained as multiple molecular fractions, all of which are serologically identical when separated and recovered from the acrylamide gel. Fig. 1 (left) is the acrylamide gel disc electrophoretic pattern of purified type 12 M protein. From preparative columns (see Materials and Methods), five fractions of M protein were isolated as illustrated. These components of type 12 M protein were administered intracutaneously to 3 individuals of predetermined reactivity. Fig. 2 shows the cutaneous reactions of 1 subject at 24 hr to a 1 μg dose of each fraction. Each

injection produced a cutaneous reaction of approximately equal intensity. Similar results were observed in the other 2 subjects.

Complete digestion of M proteins by trypsin abolished the DCH reaction in persons of known sensitivity. It was of interest to determine whether at-

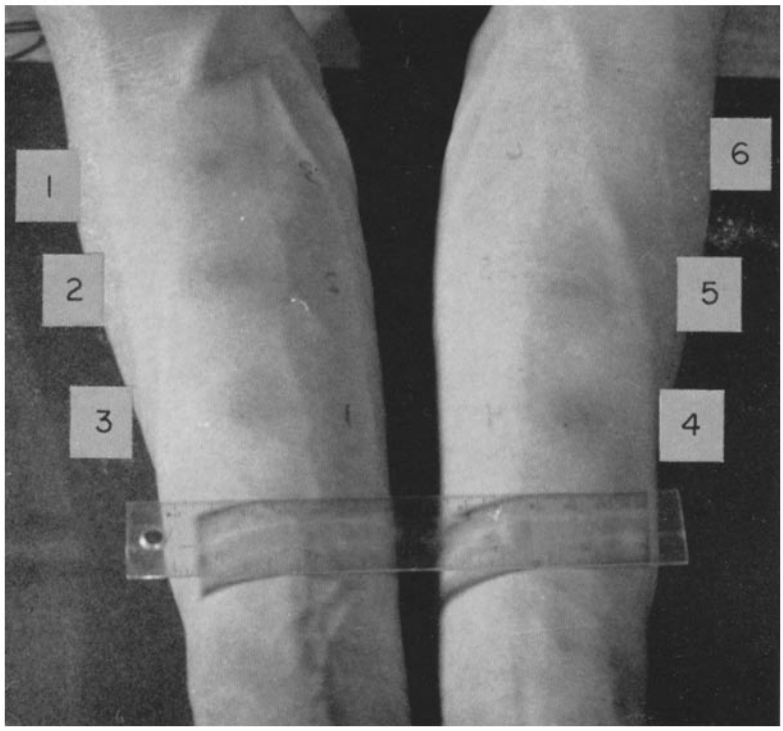


FIG. 2. Delayed cutaneous reactions at 24 hr from 1 μg doses of the five separate fractions of type 12 M protein, as shown in Fig. 1. Injection site 6 is a control preparation from a section of gel containing no M protein.

tenuated (partially degraded) M proteins would induce less cutaneous reaction while maintaining antigenic specificity. Limited tryptic digestion was carried out in the following manner. To 1 mg samples of type 12 M protein in 1.0 ml of buffered saline at pH 7.0 at 37°C was added 5 μg of crystalline trypsin (Worthington Corporation, Freehold, New Jersey) in 0.2 ml of buffer. The mixtures were incubated at 37°C and after appropriate intervals (Table III) 10 μg of crystalline soy bean trypsin inhibitor (Worthington) in 0.05 ml of buffer was added. The temperature was maintained at 37°C for an additional 10 min and then the mixture was chilled to 0°C. Table III shows the changes in antigenic activities with increased tryptic digestion. Loss of some precipitating

activity was observed after 5 min, but the residual antigen still had the capacity to partially inhibit the HA reaction. In Fig. 3 are the acrylamide gel electrophoretic patterns of the M protein fractions during the course of tryptic degradation. Within 5 min, the M protein fractions were converted to polypeptides of entirely new mobilities, yet they maintained a considerable portion of type-specific antigenic activity. When slices from the stained areas of the acrylamide gel containing the 5 min digest of M protein were placed in wells of agar plates

TABLE III

Effect of Trypsin on the Antigenicity of Type 12 M protein

Sample	Time of trypsinization	Precipitin reaction*	Hemagglutination inhibition test	DCH to 1 μg; diameter of erythema (Fig. 5)
	min		*reciprocal of dilution*‡	*cm*
1	0 (no trypsin)	++++	0	6§
2	1	++++	0	4§
3	5	+++	12,800	1.5
4	15	++	25,600	1.0
5	30	±	51,200	(omitted)
6	90	—	102,400	0.6
7	90 (no M protein)	–	102,400	0

* Precipitin reaction recorded as estimated degree of precipitation in a capillary tube with undiluted type 12 serum.

‡ Samples diluted to contain the equivalent of 80 μg of original M protein per milliliter; 0.5 ml were mixed with 0.5 ml of a 1:25 dilution of type 12 antiserum at 37°C for 30 min before titration.

§ Erythema accompanied by swelling and induration.

for immunodiffusion reactions, precipitin lines of identity were observed with type-specific antisera (Fig. 4).

3 adult volunteers of known hypersensitivity reacted approximately to the same degree when challenged intracutaneously with the trypsinized M protein samples; one result is shown in Fig. 5. Each dose contained the equivalent of 1 μg of M protein (determined before tryptic digestion). The M protein samples treated with trypsin for 5 min or longer initiated only minimal DCH. It may be assumed that the remaining low degree of DCH induced by the M proteins hydrolyzed with trypsin was due to residual polypeptides that may be seen in Fig. 3 (30 and 90 min hydrolysates).

These data demonstrate that type 12 M protein, capable of evoking a cutaneous response in hypersensitive persons, may be partially degraded with trypsin to virtually eliminate the cutaneous reaction and still maintain a portion of type-specific antigenic reactivity. The implications of these results in terms of a vaccine preparation will be discussed.

Responses of Adults to Soluble and Alum-Precipitated M Protein Vaccines.—11 adults showing no skin sensitivity to 1 μg doses of type 24 M protein were given subcutaneous injections of 20 μg of the antigen. There were no untoward local or systemic reactions resulting from these injections. The antibody responses measured by HA prior to and 10 days following the injection are shown

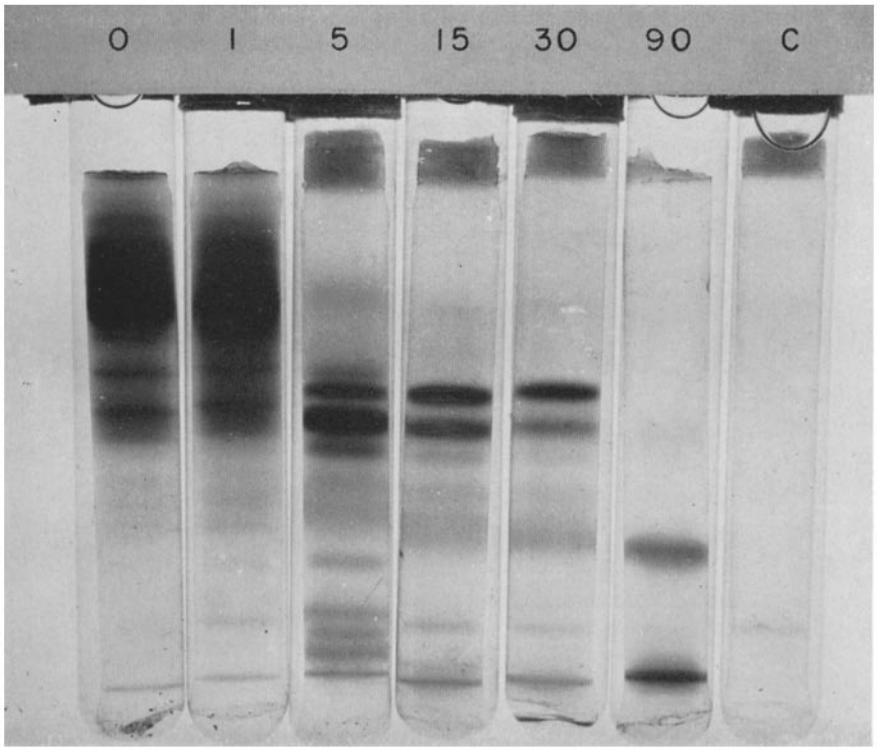
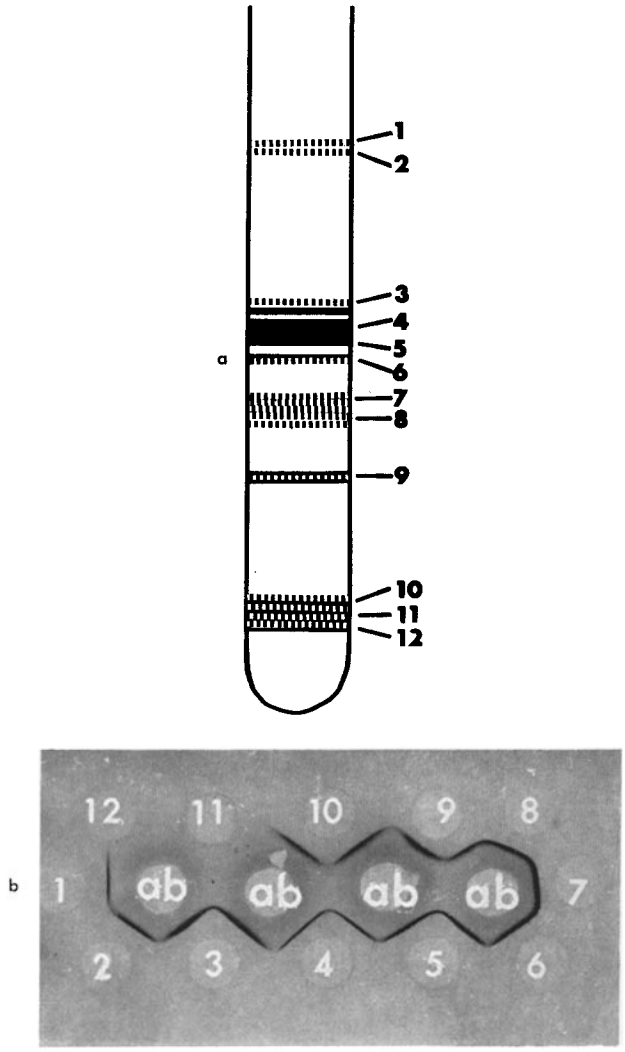


FIG. 3. Acrylamide gel electrophoretic patterns of type 12 M protein samples treated with trypsin for various intervals of time (see text). Tube C contains only the trypsin-trypsin inhibitor complex (sample 7 of Table III).

in Table IV. (The maximum response appeared between 10 and 14 days.) All 11 subjects responded, with an average threefold increment in the type-specific HA titer.

Our previous experiments with rabbits (2) demonstrated that two or three 10 μg doses of M proteins administered with adjuvants in repository injections stimulated high-titer protective antibodies. As a result of these experiences, type 12 alum-precipitated M protein (APM), composed of 10 μg of M protein adsorbed onto 2 mg of aluminum hydroxide in 0.5 ml of Ringer's lactate buffer,

was administered subcutaneously to a panel of adult volunteers previously screened for cutaneous nonreactivity to the type 12 M protein. Serum samples were obtained just prior to and at four weekly intervals after the injection. The maximum increase in titer appeared between 1 and 2 wk. Table V summarizes the results with 22 adults injected with the APM vaccine. 20 of the



FIGS. 4 *a* and 4 *b*. Diagram of stained gel electrophoretic preparation of 5 min trypsin digest of type 12 M protein. 2-mm sections from the numbered areas were placed in wells of an agar plate and reacted by immunodiffusion with type-specific serum (*ab*) placed in the inner wells.

subjects responded with antibody titer increments averaging fivefold over the preinjection level.

In a group of 19 persons who received a 1 μg dose of soluble M protein intracutaneously, but no vaccine, only 3 showed an increase in the HA titer and the over-all mean in this latter group did not increase significantly in the 2 wk interval following the skin test. A comparison of those who were skin-tested and received vaccine and those who received only the skin test is shown in the histogram of Fig. 6, in which the antibody titer increment of each subject is

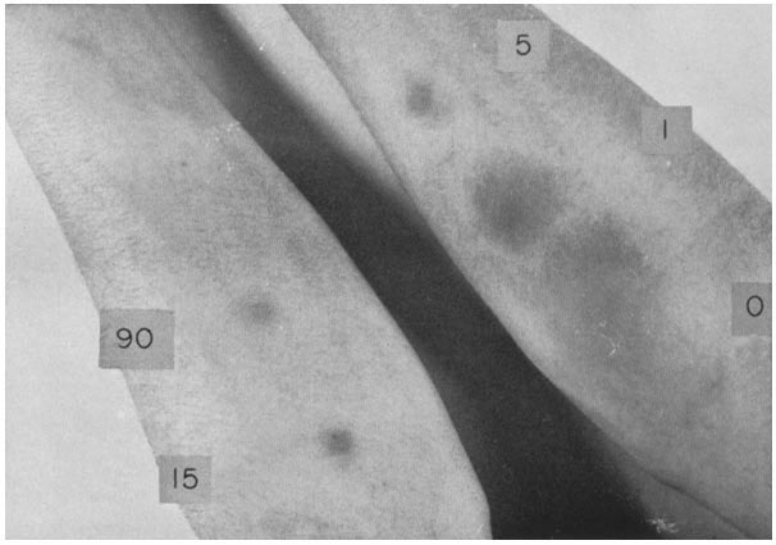


FIG. 5. Delayed cutaneous reactions (24 hr) to 1 μg samples of type 12 M protein treated with trypsin as indicated in Table III. The area distal to the 90 min reaction was the site of injection of sample 7, the trypsin-trypsin inhibitor blank (*C* of Fig. 3).

plotted. The data of these two groups are compared to illustrate that the skin-test dosage given to those subjects who received APM vaccine 24 hr later had no significant effect on the antibody responses. In the group of 19 persons who were skin-tested only, 14 exhibited DCH, but there was no correlation between the degree of DCH and the HA titers before or after the injections. However, because the subjects who exhibited DCH were omitted from the vaccine trial, there were no data to determine whether the hypersensitive state could influence the antibody response in these persons.

Sera from a number of subjects were selected for bactericidal assays (Table V). In general, sera with HA titers of 3200 or greater were bactericidal. Sera from those subjects whose HA titers increased from below this to titers above 3200 had type-specific bactericidal antibodies presumably resulting from the

APM vaccine. It was observed that postinjection sera with titers above 100,000 and occasionally 50,000 had type-specific precipitin activity when assayed by the capillary technique, whereas sera of lower titers from these subjects prior to immunization gave no visible reaction with soluble M proteins.

Relationship of M Proteins and Human Tissue.—Indirect support for the current hypothesis of the autoimmune pathogenesis of rheumatic fever and streptococcal glomerulonephritis stems from the demonstration of Kaplan and Meyeserian that a streptococcal cell wall antigen cross-reacts immunologically with human heart tissue (10). Subsequent serological studies with heart and

TABLE IV

Response to 20 μg of Type 24 M Protein in Adults

Subject	Hemagglutination titer (reciprocal of dilution)	
	Preinjection	10 day postinjection
1	200	800
2	1600	3200
3	800	3200
4	200	400
5	400	3200
6	1600	3200
7	1600	3200
8	800	3200
9	800	3200
10	1600	3200
11	400	1600
Geometric mean	700	2200

kidney tissues have corroborated this initial discovery although the various investigations do not necessarily agree on the composition or cellular origin of the streptococcal antigens (11–13). If induced autoimmune reactions prove to be etiologic factors in the sequellae of streptococcal infections, a vaccine for human use must be free of these cross-reacting antigens; this was assessed in the following ways.

17 of the highest titer antisera from our previous study of rabbits immunized with types 12 and 14 APM or M proteins in oil emulsions (2) were tested for immunological cross-reactivity with human heart tissue by fluorescent antibody techniques.[1] None of these sera showed a significant affinity for human heart tissue. However, it was reported to us by Dr. Melvin Kaplan that 1 serum

[1] These tests were performed in the laboratories of Doctors Melvin Kaplan, Earl Freimer, and John Zabriskie.

TABLE V

Antibody Responses to Alum-Precipitated Type 12 M Protein Vaccine in Adults

Subject	Hemagglutination titer × 10^{-2} (reciprocal of dilution)		Bactericidal index*	
	Preinjection	14 days postinjection	Preinjection	14 days postinjection
1	32	128		
2	64	64		
3	64	256		
4	128	2048		
5	64	512		
6	32	128		
7	0	32	0	4
8	32	64		
9	64	64		
10	128	256		
11	16	512	0	4
12	16	256	2	4
13	16	32		
14	2	8		
15	16	128	0	0
16	64	256		
17	32	1024	4	4
18	16	32		
19	16	32		
20	8	128	0	4
21	32	512	4	4
22	32	128	0	2
Mean‡	27	135	1.25	3.25

* See materials and Methods for calculation of bactericidal index.

‡ Geometric mean of HA titers and arithmetic mean of bactericidal indices.

furnished by our laboratory cross-reacted with human heart tissue. This antiserum was from a group of rabbits made hyperimmune with repeated large doses (2 mg) of type 12 M protein in mineral oil emulsion.

Rabbit antiserum against the membrane fraction of a type 25 group A streptococcus, exhibiting a strong immune reaction with human heart tissue (12) was supplied to us by Dr. Earl Freimer. This antiserum failed to agglutinate erythrocytes passively sensitized with types 12, 14, or 24 M proteins. In addition

purified type 12 M protein failed to react in immunodiffusion tests with rabbit antisera prepared against pooled human glomerular membranes[2] although the latter sera produced strong precipitin reactions with the solubilized kidney antigens (13).

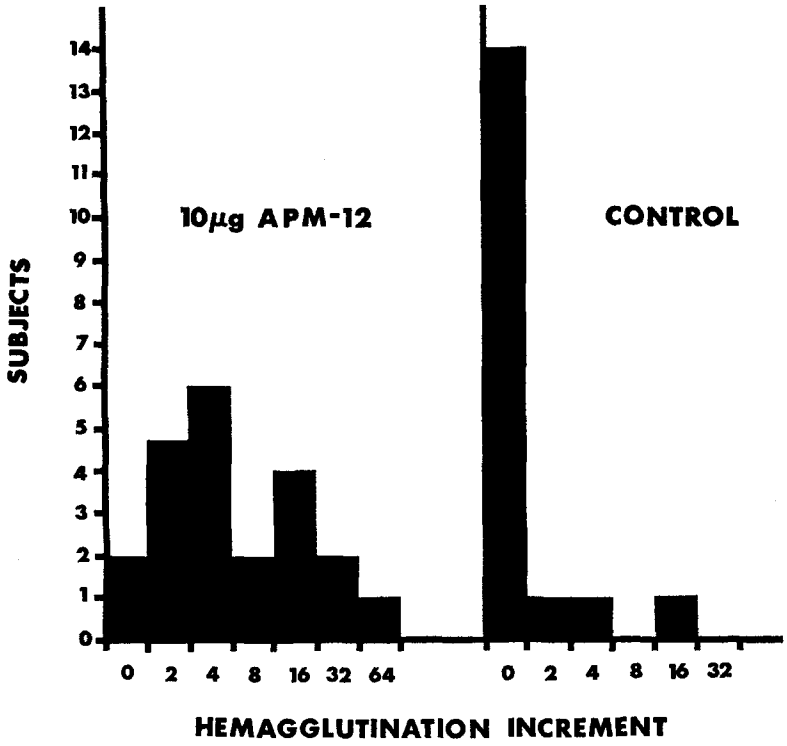


FIG. 6. Histogram of distribution of HA titer increments in 22 subjects following a subcutaneous 10 μg dose of type 12 APM (left) and a control group (right) of 19 subjects who received a 1 μg intracutaneous injection of soluble type 12 M protein (see Table V).

DISCUSSION

The delayed cutaneous reactions we have observed with purified M proteins resemble the so called "Jone-Mote" hypersensitivity. The latter is a term coined by Raffel and Newel (14) to describe a transient hypersensitivity (to streptococcal and other antigens) possibly first characterized by Jones and Mote (15). Lawrence (16) described a similar type of evanescent cutaneous sensitivity in 90% of the adult patients in his study of passive transfer reactions using partially purified M proteins. Our data show that only 8% of the infants tested

[2] This test was carried out by Dr. A. S. Markowitz.

exhibited the DCH. It can be inferred that acquired DCH in adults results from life-long exposure, clinically or subclinically, to many serotypes of group A streptococci. These conclusions are also supported by the detection of HA antibodies to M proteins in most of the adults and only a few of the infants, although the lack of reactivity in infants could also be attributed to diminished immunological responsiveness. Previous studies in humans with streptococcal culture filtrates, cellular extracts, or partially purified M proteins also showed a much higher incidence of cutaneous reactions in adults than in infants and children (17–19). However, owing to the relative severity of many of the local and systemic reactions encountered by these and other workers (20, 21), the present investigation was conducted cautiously and conservatively. On the basis of our knowledge of the antigenic purity of the M proteins used in this study, we conclude that in the present work, the tissue and humoral responses observed are type-specific indicators of streptococcal exposure.

We have not yet determined whether purified M proteins per se can induce a state of hypersensitivity in humans. Rabbits that received up to six injections of 100 μg of M protein over a period of 3 months still exhibited no DCH (6). Lawrence's study (22) in humans of the passive transfer of DCH showed no "conversions" in nonreactive subjects who received a number of doses of partially purified M proteins. In the data presented here, there appears to be no correspondence between the elicitation of a DCH response and HA titers either before or following a challenge of M protein. In future studies in larger populations, it may be revealed that M-specific DCH may be a prognostication of relative type-specific immunity to group A streptococcal infection.

The hemagglutination test using highly purified M proteins adsorbed to erythrocytes is an extremely sensitive and specific antibody assay. Our HA data on the antibody responses of adults to APM vaccine and the general correspondence with increases in the bactericidal activity of postinjection sera indicate that the HA assay may be a useful technique for measuring the efficacy of M protein vaccines in humans; similar conclusions have been borne out in our results with rabbits (2, 6).

M proteins partially degraded by trypsin retain substantial antigenicity when assayed in vitro and evoke only minimal delayed cutaneous reactions in hypersensitive persons. Acrylamide gel electrophoresis demonstrates that the large polypeptides resulting from partial hydrolysis and not residual undegraded proteins are the antigenic components. We have immunized a group of 6 rabbits with APM prepared from type 12 M protein exposed to trypsin for 5 min. HA titers after a series of three injections were comparable to those obtained with undegraded APM vaccines (2). Trypsin-attenuated M proteins may obviate a problem in the use of polyvalent APM vaccines in individuals possessing DCH to one or more serotypes.

Due to the fact that type-specific antibodies were present prior to the

antigenic stimulus, an increase in antibody titers in the majority of subjects who received soluble M proteins or APM vaccine was most likely a secondary response. Potter et al. (20) reported similar anamnestic reactions in persons injected with streptococcal cell walls. In this latter work, subjects were challenged with streptococcal walls of homologous strains isolated from specific upper respiratory infections.

An essential feature of the M protein preparations administered in the present study is the lack of toxicity in experimental animals and humans. Our preparations of soluble and APM vaccine administered to mice and guinea pigs in doses ten times greater than those introduced into humans, or several thousand times greater, calculated on a body weight basis, were nontoxic and nonpyrogenic. Schwab (23) has demonstrated that group A streptococcal cell walls contain a cytotoxic necrotizing factor, presumed to be the mucopeptide-C polysaccharide complex. It is obvious that vaccines composed of streptococcal cell walls or impure M proteins may have serious deleterious effects resulting from this necrotizing factor.

Large doses of APM vaccine administered intraperitonealy in guinea pigs produced an encapsulated granuloma which was not observed with soluble M proteins. This localized lesion in the peritoneal membrane at the site of inoculation was histologically identical to that observed by Holt (24) with alum-precipitated diphtheria toxoid. In the latter work as with APM vaccine, these nodules caused by the deposition of aluminum hydroxide were nearly resorbed within a month after the injection.

If the cross-reaction of human tissues and streptococcal antigens proves to be an etiologic factor in the postinfectious events leading to rheumatic fever and glomerulonephritis through autoimmune mechanisms, the present study most likely excludes the M proteins from culpability. The significance of the single instance of antigenic cross-reactivity of hyperimmune anti-M rabbit serum and human heart tissue is difficult to evaluate. However, in view of the absence of cross-reacting antibodies in all other rabbit sera, further testing of M protein vaccines in humans is reasonably safe. Thus, our data demonstrate that small doses of highly purified M proteins are capable of inducing type-specific anamnestic antibodies in humans under reasonably safe circumstances. Studies are now possible on larger populations to determine if immunization with multiple serotypes of M proteins will afford protection against group A streptococcal infections.

SUMMARY

Highly purified M proteins were used for determining cutaneous hypersensitivity and type-specific circulating antibodies in normal adults and infants. 80% of 91 adults and 8% of 59 infants exhibited a transient delayed cutaneous reaction to at least two of types 12, 14, and 24 M proteins. Antibodies assayed

by passive hemagglutination were observed in 90% of the adults and 13% of the infants.

Vaccines of 10 μg of alum-precipitated M protein or 20 μg of the soluble antigen were administered to adults not exhibiting delayed hypersensitivity. Within 2 wk hemagglutination titers increased significantly in 31 of 33 subjects. Preimmunization antibody levels indicated that these responses were probably anamnestic reactions from previous exposures to homologous serotypes of group A streptococci. Sera exhibiting large increments in antibody titers resulting from M protein inoculations also had type-specific bactericidal properties. "Attenuated" M proteins, produced by partial degradation with trypsin induced only minimal cutaneous reactions in hypersensitive adults, but still retained most of the antigenic specificity when assayed in vitro and in vivo.

The utility of M protein vaccines for human use is discussed in reference to the low incidence of cutaneous hypersensitivity in infants, the potentials of polyvalent attenuated M protein vaccines and the apparent absence of immune cross-reactivity between pure M proteins and human heart and kidney tissues.

The authors express their appreciation to Dr. Melvin Kaplan, Western Reserve University, Doctors Earl Freimer and John Zabriskie of The Rockefeller University and Dr. A. S. Markowitz of the Hektoen Institute for their cooperation in this study. We also thank Mrs. Tamara Smoot and Dr. Priscilla Kao for their expert technical assistance.

We are grateful to Doctors Jacob Holper and Donald Stoppel of Abbott Laboratories, Inc., for their cooperation in the safety testing of the M protein vaccines, and to Dr. Howard M. Jacobs for his aid in part of the clinical testing.

BIBLIOGRAPHY

1. Lancefield, R. C., Current knowledge of type-specific M antigens of group A streptococci, *J. Immunol.*, 1962, **89**, 307.
2. Fox, E. N., and Wittner, M. K., Antigenicity of the M proteins of group A hemolotyic streptococci. II. Antibody response in rabbits to vaccines prepared with oil emulsions and aluminum hydroxide, *J. Immunol.*, 1966, **97**, 86.
3. Fox, E. N., and Wittner, M. K., The multiple molecular structure of the M proteins of group A streptococci, *Proc. Nat. Acad. Sc.*, 1965, **54**, 1118.
4. Public Health Service Regulations for Biological Products, Title 42, Part 73, 1965, United States Public Health Service Regulations No. 73–74, General Safety, p. 14.
5. Swift, H. F., Wilson, A. T., and Lancefield, R. C., Typing group A hemolytic streptococci by M precipitin reactions in capillary pipettes, *J. Exp. Med.*, 1943, **78**, 127.
6. Fox, E. N., Antigenicity of the M proteins of group A hemolytic streptococci, *J. Immunol.*, 1964, **93**, 826.
7. Lancefield, R. C., Differentiation of group A streptococci with a common R antigen into three serological types, with special reference to the bactericidal test, *J. Exp. Med.*, 1957, **106**, 525.
8. Stollerman, G. H., Kantor, F. S., and Gordon, B. D., Accessory plasma factors

involved in the bactericidal test for type-specific antibody to group A streptococci. I. Atypical behavior of some human and rabbit blood, *J. Exp. Med.*, 1958, **108,** 475.

9. Lewis, U. J., and Clark, M. O., Preparative methods for disc electrophoresis with special reference to the isolation of pituitary hormones, *Anal. Biochem.*, 1963, **6,** 303.
10. Kaplan, M. H., and Meyeserian, M., An immunological cross-reaction between group A streptococcal cells and human heart tissue, *Lancet*, 1962, **1,** 706.
11. Kaplan, M. H., and Suchy, M. L., Immunological relation of streptococcal and tissue antigens. II. Cross-reaction of antisera to mammalian heart tissue with a cell wall constituent of certain strains of group A streptococci, *J. Exp. Med.*, 1964, **119,** 643.
12. Zabriskie, J. B., Freimer, E. H., and Seegal, B., An immunological relationship between streptococcal membranes and human heart tissue, *Fed. Proc.*, 1964, **23,** 343.
13. Markowitz, A. S., and Lange, C. F., Jr., Streptococcal related glomerulonephritis. I. Isolation, immunochemistry and comparative chemistry of soluble fractions from type 12 nephritogenic streptococci and human glomeruli, *J. Immunol.*, 1964, **92,** 565.
14. Raffel, S., and Newel, J. M., The "delayed hypersensitivity" induced by antigen-antibody complexes, *J. Exp. Med.*, 1958, **108,** 823.
15. Jones, T. C., and Mote, J. R., The phases of foreign protein sensitization in human beings, *New England J. Med.*, 1934, **210,** 120.
16. Lawrence, H. S., Transfer of skin reactivity to streptococcal products, *in* Streptococcal Infections, (M. McCarty, editor), New York, Columbia University Press, 1954, 152.
17. Mackenzie, G. M., and Hangar, F. M., Jr., Allergic reactions to streptococcus antigens, *J. Immunol.*, 1927, **13,** 41.
18. Howell, K. M., and Corrigan, M., Skin reactions with bacterial filtrates of anhemolytic streptococcus, hemolytic streptococcus and *B. typhosus*, *J. Infect. Dis.*, 1928, **42,** 149.
19. Schmidt, W. C., Type-specific antibody formation in man following injection of streptococcal M protein, *J. Infect. Dis.*, 1960, **106,** 250.
20. Potter, E. V., Stollerman, G. H., and Siegel, A. C., Recall of type-specific antibodies in man by injections of streptococcal cell walls, *J. Clin. Inv.*, 1962, **41,** 301.
21. Wolfe, C. K., Jr., Hayashi, J. A., Walsh, G., and Barkulis, S. S., Type-specific antibody response in man to injections of cell walls and M protein from Group A, type 14 streptococci, *J. Lab. and Clin. Med.*, 1963, **61,** 459.
22. Lawrence, H. S., The cellular transfer in humans of delayed cutaneous reactivity to hemolytic streptococci, *J. Immunol.*, 1952, **68,** 159.
23. Schwab, J. H., Biological properties of streptococcal cell-wall particles. I. Determinants of the chronic nodular lesion of connective tissue, *J. Bacteriol.*, 1965, **90,** 1405.
24. Holt, L. B., Developments in Diphtheria Prophylaxis, London, William Heinemann, Ltd., 1950, 90.