HUMAN IMMUNE RESPONSE TO IMMUNIZATION WITH A STRUCTURALLY DEFINED POLYPEPTIDE FRAGMENT OF STREPTOCOCCAL M PROTEIN*

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The mechanism of the pathogenesis of rheumatic fever and rheumatic carditis remains unknown (1). Efforts to vaccinate against group A streptococci have been hampered by toxicity of most preparations of M protein and the fear that streptococcal vaccines may contain some of the antigens shown to be cross-reactive with heart tissues (2–4) and, therefore, theoretically may trigger, rather than prevent, rheumatic fever and carditis.

It has been recognized for a long time that the resistance of group A streptococci to phagocytosis is largely attributable to the presence on the surface of virulent organisms of a type-specific antigen, the M protein. Hosts who are immune to a particular M serotype of streptococci possess antibodies that render organisms bearing the homologous type of M protein on their surfaces susceptible to ingestion and killing by host phagocytes (5). Efforts to purify the M antigen in the past, however, have failed to separate heterologous protein antigens from the type-specific determinant of M protein (6–11). Moreover, after extensive purification, the M antigen often became less immunogenic and was of questionable value in affording protective immunity (12).

In our search for more effective methods for the extraction and purification of M protein for use as vaccines, we found that the antigen could be extracted in satisfactory yields by subjecting intact streptococci to limited digestion with pepsin (13). Upon purification of the peptic extracts of types 6 and 24 M protein (pep M)[1] by ammonium-sulfate precipitation, ion-exchange chromatography, and isoelectric focusing, the heterologous, heart-reactive, and toxic materials were readily separated from the type-

* Supported by program-directed research funds from the United States Veterans Administration and by research grants AI-10085 and AI-13550 from the United States Public Health Service.

Presented in part at the annual meeting of the Association of American Physicians, Washington, D. C., May 1979.

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[1] *Abbreviations used in this paper:* CF, complement fixation; CFU, colony-forming units; DCH, delayed cutaneous hypersensitivity; ELISA, enzyme-linked immunosorbent assay; NTSM, non-type-specific M; PBS, phosphate-buffered saline; PBS-Tween, PBS supplemented with 0.05% Tween and 0.02% NaN_3; pep M, purified peptic extracts of types 6 and 24 M protein; pep M6, type 6 M protein pepsin extracts; pep M24, type 24 M protein pepsin extracts; saline, 0.85% solution of NaCl; saline-Tween, 1 ml of 0.15 M NaCl containing 0.05% Tween; SDS, sodium dodecyl sulfate.

J. Exp. Med. © The Rockefeller University Press · 0022-1007/79/10/0862/16 $1.00
Volume 150 October 1979 862–877

specific M antigen (14). Physiochemical analysis and partial amino acid sequence analysis indicated that these fragments of M protein molecules are composed of a single, unbranched polypeptide chain (15).

The type 24 pep M protein (pep M24) molecule was cleaved with cyanogen bromide into seven peptides, each of which, after purification, inhibited type-specific opsonic antibody, and therefore, each peptide presumably contained the protective determinant. Partial sequence analyses of the seven peptides suggested that the type-specific antiopsonic determinant resides in a repeating amino acid sequence in the intact M protein molecule (16). When incorporated into complete Freund's adjuvant and injected into laboratory animals, the intact pep M24 evoked type-specific protective antibodies. The immune sera were free of non-type-specific antibodies that are cross-reactive with M-associated antigens (14, 15). Furthermore, they were free of cross-reactivity with human heart tissue.

Having established that the purified pep M protein was immunogenic and free of immunological cross-reactivity with heart tissue, we initiated studies of the immunogenicity of this unique antigenic molecule in man. In this paper, we describe the type-specific immunogenic properties of alum precipitates of pep M24, first in laboratory animals and then in man. Our data indicate that immunization with the adjuvant mixture induces type-specific protective immunity in laboratory animals. We show further that the pep M24 vaccine is well-tolerated in doses sufficient to produce similarly type-specific, primary opsonic and protective antibody responses in man.

Materials and Methods

Purification of Streptococcal M Protein. M protein prepared from limited peptic digests of whole, group A streptococci was obtained in pure form as judged by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, quantitative amino acid analysis, and Edman degradation, by methods previously described (14, 16). The streptococci were grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) in 60-liter batches for 16 h, sedimented by centrifugation, washed twice in 20 mM phosphate/0.15 M NaCl, pH 7.4 (PBS), and once in 67 mM phosphate, pH 5.8. The organisms were finally resuspended in three volumes of the buffer at pH 5.8 containing 50 μg of pepsin/ml (Worthington Biochemical Corp., Freehold, N. J.) and incubated for 1 h at 37°C. Digestion was stopped by adding 7.5% $NaHCO_3$ to raise the pH to 7.5. After incubation for 1 h at 25°C, the organisms were sedimented by centrifugation at 10,000 *g* (DuPont Instruments-Sorvall Operations, DuPont Co., Newtown, Conn.) for 20 min, and the cells were extracted a second time with pepsin under identical conditions. The supernates were combined, filtered through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.), dialyzed against PBS, lyophilized, redissolved in 10 ml distilled water, further dialyzed against distilled water, and relyophilized. The lyophilized material containing the M protein antigens was redissolved in PBS and precipitated by slowly adding saturated ammonium sulfate. The type 24 M antigen was precipitated at 30% saturation, whereas the type 6 M antigen was precipitated between 30 and 60% saturation with ammonium sulfate. The ammonium sulfate precipitates were redissolved, digested with ribonuclease, dialyzed, lyophilized as described by Lancefield and Perlmann (17), and designated pep M.

The partially purified pep M preparations were redissolved in 50 mM Tris-HCl buffer at pH 7.5 and fractionated by ion-exchange chromatography on columns of quarternary aminoethyl Sephadex A-50 (Pharmacia Fine chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) (14). The material eluted at a salt concentration of 0.2 M NaCl (18). It was homogeneous on SDS-gels (19) and by quantitative amino acid analysis (20); the final isoelectrofocusing step (14), therefore, was omitted. The elution peak containing the homogeneous pep M in each case was dialyzed against distilled water and lyophilized. The purified material was assayed for the presence of the type-specific anti-opsonic and immunoprecipitating antigen by the methods previously described in detail (14, 16).

Preparation of pep M Antigens for Injection. For skin testing, the lyophilized pep M preparations were dissolved at the appropriate concentrations in sterile water for injection (without preservatives). Under sterile precautions, the pep M solutions were sterilized by passing through a 0.45-μm membrane filter and packaged in sterile multiple-dose vials and lyophilized. Just before use, the lyophilized antigens were dissolved in the appropriate amount of sterile 0.85% NaCl for injection. The skin test dose ranged from 0.1 to 1.0 μg in a vol of 0.1 ml injected intracutaneously on the volar surface of either forearm.

For vaccination, the lyophilized pep M preparations were precipitated in aluminum hydroxide by the method of Chase (21). All materials except the sodium hydroxide were sterilized by membrane filtration and the entire precipitation and packaging procedures were carried out in a sterile room in the Division of Parenteral Medications under the direction of Dr. Kenneth Avis at the University of Tennessee College of Pharmacy, Memphis, Tenn. 10 mg of pep M in 6 ml of 0.85% NaCl (saline) was mixed with 3.2 ml of a 10% solution of aluminum potassium sulfate. The solution was immediately neutralized during thorough mixing by the dropwise addition of 1.4 ml of 1.0 N NaOH followed by dropwise addition of 0.4 N NaOH to reach a pH of 6.0. Sufficient additional saline was then added to make a total vol of 10 ml. After 1 h, the precipitate was centrifuged at 1,000 *g* for 10 min and resuspended in 25 ml of sterile saline for a final concentration of 200 μg of M protein per 0.5 ml of vaccine dose. The alum-precipitated vaccine was then packaged in sterile multiple-dose vials and stored at 4°C until used. Measurement of the protein content of the first supernate after alum precipitation by the method of Lowry et al. (22) revealed that >95% of the pep M had been adsorbed to the aluminum gels.

Both the antigens used for skin testing and the alum-precipitated antigens were tested for sterility, pyrogenicity, and toxicity according to the protocols of the Division of Biological Standards of the U. S. Public Health Service (23). All preparations used were within acceptable limits.

Volunteers. 12 healthy male and female volunteers between the ages of 22 and 58 yr (mean age, 29 yr) were selected for the human studies. Individuals with histories or evidence of heart or kidney disease, rheumatic fever, or atopic allergies were excluded as were those who had type-specific antibodies to types 6 or 24 streptococci. All individuals who volunteered for the study were informed of the potential hazards in compliance with the standards of the U. S. Public Health Service, of the Human Use Committee of the University of Tennessee Center for the Health Sciences, and of the Memphis Veterans Administration Hospital.

All selected individuals were skin tested with 0.1- to 1.0-μg doses of pep M protein injected intracutaneously in a total vol of 0.1 ml of saline. Skin-test-negative individuals who were chosen for the vaccination studies received the alum-precipitated type 24 vaccine in doses of 100–200 μg of protein injected subcutaneously in the upper lateral aspect of the arm. Sera were collected from all vaccinees immediately before the first dose and at 2-wk intervals thereafter and were assayed for the presence of type-specific, non-type-specific, and heart cross-reactive antibodies. Of the 12 volunteers, 2 (G. S. and R. J.) were the subjects of a pilot course of gradually increasing doses of pep M24 vaccine. Subsequently, 10 additional volunteers were started on an immunization schedule consisting of three doses of 200 μg of pep M every 2 wk, followed by a fourth 200-μg dose 3–4 mo after the third dose. Of the 10, 3 received only the first two doses because of minor reactions (Results). A fourth was unavailable to receive the last dose. Thus, including the two pilot volunteers, eight individuals completed the protocol of four doses.

Type-specific Opsonic Antibody Assays. In vitro timed phagocytosis assays for type-specific opsonic M antibody were performed as described (14, 16, 24). The assay mixtures consisted of 0.4 ml fresh human blood supplemented with 10 U/ml of heparin, 0.05 ml of a standard suspension of streptococci, and 0.05 ml of test serum. The ratio of streptococcal colony-forming units (CFU) per leukocyte was ≅10:1. The percentage of neutrophilic leukocytes that ingested streptococci (percentage of phagocytosis) was estimated by microscopic counts of stained smears prepared from the assay mixture after 30 min of incubation. The opsonic antibody titer is expressed as the reciprocal of the highest twofold dilution of test serum that in three separate tests of each serum-produced phagocytosis of 10% or greater when phagocytosis of the same organisms in the presence of nonimmune rabbit serum was < one-half that of the test serum in

each assay. Type specificity was demonstrated by failure of the immune sera to opsonize heterologous M serotypes of group A streptococci.

Bactericidal tests were performed using the same test mixtures as described above except that a smaller number of streptococci (ranging from 2 to 50 CFU per 0.4 ml of fresh blood) were added to the assay mixtures. The test mixtures were tumbled end-over-end at 8 rpm at 37°C for 3 h and finally mixed with melted sheep blood agar to prepare pour-plates for colony counts as previously described (7).

Long-Chain test. The test was performed according to a method previously described (25).

Tests for Type-specific Antibody Responses by Enzyme-linked Immunosorbent Assays (ELISA). ELISA for anti-M antibodies were performed according to the principles of Engvall and Perlmann (26) and as described by Russell et al. (27). 1-ml samples of M protein antigen dissolved at a concentration of 5 μg/ml in 0.1 M carbonate buffer, pH 9.6, and supplemented with 0.02% NaN_3, were incubated for 3 h at 37°C in a series of 12- × 75-mm polystyrene tubes (Falcon Labware, Div. of Becton, Dickinson, & Co., Oxnard, Calif.). After washing three times with 1 ml of 0.15 M NaCl containing 0.05% Tween 20 (saline-Tween; polyoxethylene sorbitan monolaurate, Sigma Chemical Co., St. Louis, Mo.), the antigen-coated tubes were incubated with 1-ml samples of immune serum diluted in PBS supplemented with 0.05% Tween and 0.02% NaN_3 (PBS-Tween) for 3 h at 37°C. After washing again as above, 1 ml of alkaline phosphatase-conjugated rabbit anti-human IgG (Microbiological Associates, Walkersville, Md.) diluted in PBS-Tween was added to each tube and incubated overnight at 37°C. The tubes were again washed three times with saline-Tween, after which, 1 ml of a 1 mg/ml solution of *p*-nitrophenylphosphate (Sigma Chemical Co.) in 0.1 M carbonate buffer, pH 9.6, containing 1 mM $MgCl_2$ was added. The enzyme reaction was allowed to proceed for 1 h at 37°C. The reaction was stopped by adding 0.1 ml 1 N NaOH. Absorbance was read at 400 nm in a Coleman junior spectrophotometer (Perkin-Elmer, Corp., Instrument Div., Norwalk, Conn.). The titer of the test serum was recorded as the inverse of the last serum dilution which gave an absorbance of >0.1.

Assays for Non-Type-specific and Tissue Cross-Reactive Antibodies. Complement fixation tests were performed by a micro technique as previously described (28). The non-type-specific antibody titers were expressed as the reciprocal of the highest dilution of immune serum that inhibited lysis of antibody-coated erythrocytes by 50% in the presence of an acid-extracted type 30 M protein and complement.

Immune sera were tested for the presence of heart cross-reactive antibodies as previously described (18). Frozen sections (4-μm thick) were prepared from fresh rabbit heart tissue and were stored at −80°C. The tissue sections were incubated with immune serum for 30 min at 25°C, washed three times with PBS, treated with fluorescein-labeled goat anti-human IgG (N. L. Cappel Laboratories, Cochranville, Pa.) for 30 min at 37°C, again rinsed three times in PBS and finally mounted with one drop of glycerol-glycine buffer at pH 7.6 and a glass coverslip. The treated sections were examined for fluorescence with a Leitz microscope (E. Leitz, Inc., Rockleigh, N. J.).

Analytical Methods. Protein concentrations in the purified pep M preparations were determined by quantitative amino acid analysis as described (14, 16). Samples were hydrolyzed in twice-distilled, constantly boiling HCl under an atmosphere of nitrogen for 24 h at 108°C. The hydrolyzed samples were then analyzed on a Beckman 121 automatic amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.) by a single-column technique with a four-buffer elution system (20).

SDS-polyacrylamide gel electrophoresis was performed in 10% gels according to the method of Weber and Osborn (19).

Streptococcal Antisera and Animal Immunization Studies. Antisera to whole group A streptococci were prepared by immunizing rabbits with heat-killed or ultraviolet-irradiated organisms as described (24). The animal immunogenicity of the purified pep M antigens precipitated with alum was studied by injecting mice subcutaneously with 10-μg doses and rabbits with 50-μg doses of pep M at 2-wk intervals for a total of three doses. The rabbits were bled at 2-wk intervals and the mice were challenged intraperitoneally 2 wk after the last immunizing dose with various doses (ranging from 200 to 2×10^6 CFU) of homologous or heterologous serotypes of group A streptococci suspended in a total vol of 0.5 ml PBS. The survivals were recorded

TABLE I

Indirect Bactericidal Tests with the Vaccine Strain of the Sera of Rabbits Immunized with Alum-precipitated pep M24 Vaccine

Time serum sample collected*	Number of CFU after 3-h incubation in test mixture with initial inoculum of:	
	9 CFU	3 CFU
Preimmunization	>2,000	1,280
2 wk	>2,000	1,500
3 wk	1,235	815
5 wk	340	375
6 wk	340	125
8 wk	80	90

* The sera of two rabbits were pooled for each sample. The indicated times of sample collection are the intervals after the first dose of 50 μg of pep M24. Booster doses of 50 μg were given at weeks 2 and 4. CFU, colony forming units.

over a 7-d period. Passive mouse protection tests were performed in the same fashion except that instead of vaccination, the mice were injected intraperitoneally with 0.2 ml of immune or nonimmune serum and then challenged by the same route 24-h later with homologous or heterologous serotypes of streptococci. The data were analyzed statistically by the method of Reed and Muench (29).

Results

Structure of pep M Protein Molecules. Analysis of each batch of the pep M24 vaccine by SDS-polyacrylamide gel electrophoresis, by quantitative amino acid analysis, and Edman degradation confirmed the chemical homogeneity of the pep M24 antigen. The partial amino acid sequences of the M24 molecule were shown elsewhere (16). A comparison by computor data analysis of the parts of the molecule already sequenced with >100,000 other proteins whose amino acid sequences have been reported (30) revealed only minor homologies; the longest matching sequences included a hexapeptide, Glu-Ala-Glu-Lys-Ala-Ala, a repeating segment in pep M24, which is shared with tropomyosin, a protein of rabbit skeletal muscle. The pentapeptide, Ala-Glu-Lys-Ala-Ala which is encompassed in the above hexapeptide is also shared with the hemoglobin β-chains of the rat, the mouse, and the yellow-cheeked vole.

Aluminum-hydroxide gels have been well-tolerated in man and have been shown to serve as good adjuvants for the stimulation of the immune responses to other vaccine antigens (21). Therefore, the pep M24 antigen was incorporated into aluminum gels for the following studies.

Immunogenicity of Alum Precipitates of pep M24 in Rabbits. In vitro phagocytosis tests of sera obtained from two rabbits immunized twice a week with 50-μg doses of pep M24 in alum indicated that both animals developed peak levels of type 24 opsonic antibodies 6 wk after the initial immunizing dose. Indirect bactericidal tests for M24 antibodies confirmed these results. The number of viable streptococci capable of surviving incubation in rotated test mixtures containing fresh human blood, the vaccine strain in type 24 streptococci, and rabbit immune serum decreased with increasing time intervals after the first immunizing dose (Table I).

Assays of the antisera in immunofluorescence tests made with frozen sections of

TABLE II

Type-specific Protective Immunogenicity of Alum Precipitates of pep M24 in Mice Challenged with Type 6 or Type 24 Streptococci

Mice immunized with:	LD_{50} in mice challenged with:*	
	Type 6 streptococci	Type 24 streptococci
PBS-alum (control)	<200	<200
pep M24-alum	<200	>2,000,000

* The mice were immunized and challenged with streptococci as described in the text. The number of deaths was recorded over a period of 7 d.

human heart tissue confirmed that heart cross-reactive antibodies were absent. These results are consistent with previous studies indicating that sera from animals hyperimmunized with pep M24 in complete Freund's adjuvant were free of heart cross-reactive antibodies (18).

Protective Immunogenicity of Alum-precipitated pep M24 in mice. To assess more directly the protective properties of the pep M24 vaccine, 30 mice were immunized twice a week with 10-μg doses subcutaneously of pep M24 precipitated in alum. 30 control mice were injected in the same way with alum alone. 2 wk after the last injection, 15 mice in each group were challenged with the vaccine strain of type 24 streptococci, and the remaining 15 in each group were challenged with a heterologous serotype 6 strain of streptococci. The animals were further divided into subgroups of three mice and injected intraperitoneally with various doses of streptococci ranging from 200 to 2,000,000 CFU in a total vol of 0.5 ml. None of the unimmunized mice survived challenge infections with as few as 200 CFU of either serotype of streptococcus (Table II), nor were there any survivors among the pep M24-immunized mice challenged with type 6 streptococci. In contrast, all but one of the pep M24-immunized mice survived challenge infections of the homologous vaccine strain in doses as high as 2,000,000 CFU.

These results indicated that the pep M24 molecule precipitated in aluminum hydroxide was immunogenic, and evoked type-specific protective immunity in laboratory animals. Together with the knowledge that the pep M24 molecule was structurally unique and did not induce non-type-specific (14, 15) nor heart cross-reactive antibodies, these results encouraged us to pursue pilot studies of its immunogenicity in man.

Human Skin tests and Determination of pep M24 Vaccine Dose. Skin tests of all 37 adult volunteers tested with 1-μg intradermal doses of pep M24 were negative, and intradermal doses as high as 40 μg were well-tolerated. This contrasted with almost universally positive skin reactions ranging from immediate, to delayed-type reactions to 1-μg doses of M protein vaccines prepared from conventional HCl extracts of group A streptococci (31).

2 of the 37 adult volunteers were chosen to determine the dose of alum-precipitated M antigen required to induce a type-specific opsonic antibody response. Initially, 100-μg doses were administered subcutaneously at ≅1-mon intervals. Opsonic antibodies, however, did not become detectable even after three such doses. The dose of M protein, therefore, was increased to 200 μg and given at 2-wk intervals. A brisk opsonic antibody response was observed 2 wk after the first 200-μg dose, reaching a titer of

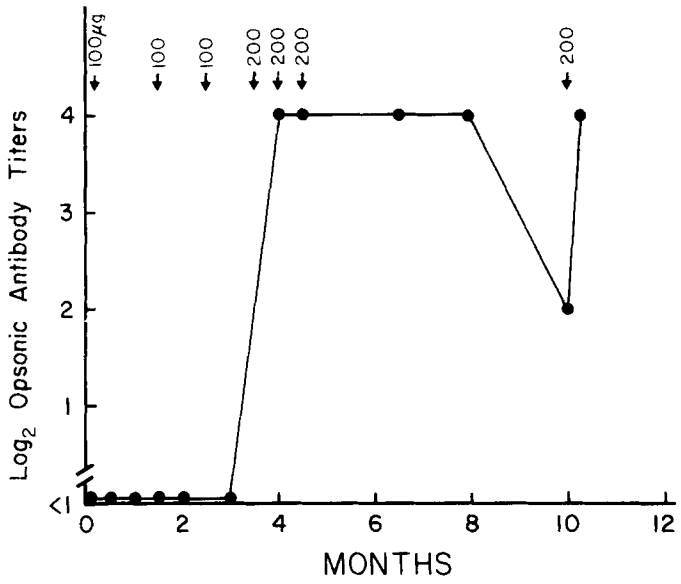

FIG. 1. Opsonic antibody response of one of the human volunteers used to determine the dose of pep M24 vaccine needed to produce a primary immune response. The doses and intervals of injection of the alum-precipitated pep M24 are indicated by the arrows.

1:16 (Fig. 1). Opsonic antibody was still present at a titer of 1:4 14 wk later, and a reinforcing dose of 200 μg boosted the antibody titer to 1:16. Neither of these two vaccinees had any complaints except for mild local irritation after receiving the subcutaneous injections of vaccine.

Type-specific Opsonic Antibody Responses in Human Volunteers. Based on the above results, another ten of the skin test-negative volunteers were immunized with 200-μg doses of alum-precipitated M protein at 2-wk intervals. The response of one of these vaccinees is depicted in Fig. 2. Low levels of type 24 opsonic antibody appeared at 4 wk and reached a peak titer of 1:32 at 8 wk, similar to the response seen previously in rabbits (above). No opsonic antibodies were formed against the heterologous type 6 streptococci during the course of immunization with pep M24.

8 of the 10 vaccinees developed opsonic antibodies 6 wk after the initial dose of vaccine (Table III). The number showing detectable opsonic antibodies fell to 3 of 10 at 10 wk.

Of the total of 12 volunteers, 9 had no significant local or systemic reactions to injections of the pep M24-alum vaccine. Of the remaining three, one had a transient low-grade fever (38°C) after the second dose, one had transient (≅24 h) local induration, and one had a transient mild exacerbation of a chronic rash. These three volunteers did not receive a third dose. These results indicated that the pep M24-alum vaccine was well-tolerated in sufficient doses to produce a primary opsonic antibody response.

Development of Type-specific Delayed Cutaneous Hypersensitivity in pep M24 Vaccinees. Skin tests of the immunized individuals showed that 10 of the 12 converted to positive delayed-type reactions (maximum responses at 24 h) to the homologous type 24 M protein (Table IV). Only four converted to positive reactions when skin tested with type 6 M protein. These results indicate that the production of hypersensitivity was

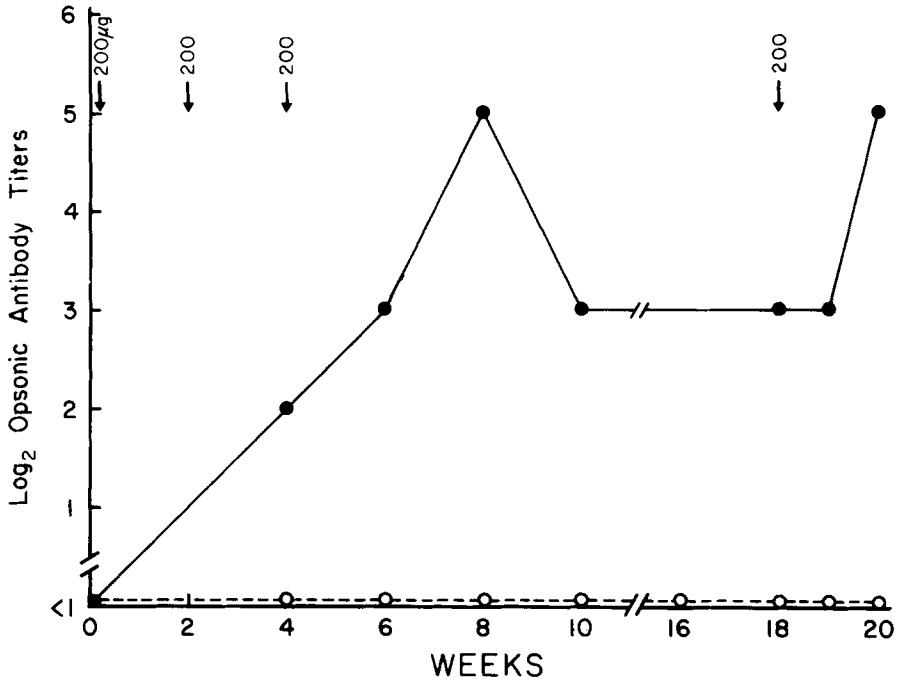

FIG. 2. Type-specific opsonic antibody response in a human volunteer injected with three 200-μg doses of pep M24 in alum at 2-wk intervals (arrows). (●) type 24 streptococci, (○) type 6 streptococci.

TABLE III

Immunogenicity of pep M24 in Humans as Determined by Opsonic Antibody Responses

Dose of pep M24*	Time	Opsonic antibodies‡ (No. positive/No. tested§)
μg	wk	
200	0	0/10
200	2	ND
200‡	4	1/10
—	6	8/10
—	8	8/10
—	10	3/10

ND, not done.

§ Data on first two pilot volunteers not included in this table (text and Fig. 2).

* M protein was injected intramuscularly as an alum precipitate in a total vol of 0.5 ml (Materials and Methods). 3 of the 10 volunteers did not receive a third dose (text).

‡ Opsonic antibodies against type 24 streptococci were assayed in timed phagocytosis tests as described in Materials and Methods. A positive test is defined as one in which phagocytosis after 30 min at 37°C is 10% or greater when phagocytosis of the same organisms in the presence of preimmune serum from the same individual is equal to one-half or less than that of the test serum.

mostly type specific but also suggested that types 6 M and 24 M protein molecules may share certain minor determinants. One individual who had measurable M24 antibodies in his serum before immunization and was therefore excluded from the study showed positive delayed-type skin reactions to pep M24.

TABLE IV

Development of Type-specific Delayed-Type Skin Reactions in Human Volunteers Immunized with pep M24 Vaccine

Skin test antigen*	Skin reactions‡ (No. positive/No. tested)	
	preimmunization	postimmunization
pep M24	0/12	11/12
pep M6	0/12	4/12

* The purified antigens (1.0 μg) were injected in a total vol of 0.1 ml of sterile saline. Control sites were injected with the same volume of saline without antigen.

‡ Skin tests were performed before immunization and 4–10 wk after the initial vaccine dose. The numbers represent accumulated positive reactions over the accumulated number of individuals tested. A positive reaction is defined as induration of 10 mm in Diam or greater, 24 h after the intradermal injection.

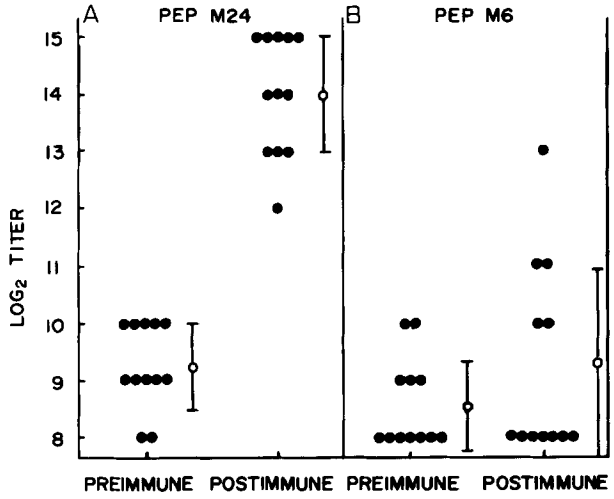

FIG. 3. Type-specific immune response to injections of alum-precipitated pep M24 in normal human volunteers as measured by ELISA. The vertical bars indicate the mean and one SD from the mean of the preimmune and postimmune values obtained using pep M24 (A) or pep M6 (B) as antigen.

ELISA of the Antibody Response to the pep M24 Vaccine in Man. To determine the total humoral antibody response to immunizing doses of the M24 molecule, the preimmune and postimmune sera of the human vaccinees were assayed by a sensitive ELISA method using either the homologous pep M24 or the heterologous type 6 M protein peptic extract (pep M6) as antigens. The total humoral antibody responses were almost entirely specific for the pep M24 vaccine (Fig. 3). Only one vaccinee showed an antibody rise of more than two tubes against the pep M6 molecule, whereas all the vaccinees developed significant antibody rises against the pep M24 molecule.

Development of Type-specific Bactericidal and Mouse Protective Antibodies in the Sera of Vaccinees. To demonstrate that the immune sera of the human vaccinees contained protective antibodies against the vaccine strain of type 24 streptococci, we performed indirect bactericidal and mouse protection tests. By the indirect bactericidal test, five of eight immune sera tested from the vaccinees inhibited the growth of type 24

TABLE V

Inhibition of Growth of Type 24 Streptococci in Fresh, Normal Human Blood in the Presence of the Sera of pep M24 Vaccinees *

Vaccinees	No. of CFU in presence of sera:‡	
	preimmune	immune
D. L.	>1,500	61
R. F.	>1,500	6
S. G.	>1,500	0
B. M.	>1,500	>1,500
H. C.	>1,500	>1,500
S. C.	>1,500	>1,500
G. S.	>1,500	0
R. J.	>1,500	0
Rabbit (pooled serum)§	>1,500	90

* Includes the eight volunteers who completed the full course of immunization (four doses, each of 200 μg).

‡ The test mixtures consisted of 0.4 ml fresh, nonimmune human blood, 0.05 ml human or rabbit serum, and 0.05 ml of a suspension of ≅10 CFU of type 24 streptococci. The mixtures were incubated at 37°C by rotation at 8 rpm for 3 h. The surviving CFU were determined by preparing pour-plate cultures from the test mixtures.

§ Pool of two sera obtained from rabbits immunized with alum-precipitated pep M24 (see Table I).

TABLE VI

Long-Chain Formation of M24 Streptococci in the Presence of Vaccinee's Sera *

Vaccinees	Mean No. of streptococci in chain ± SEM	
	Preimmune	Postimmune
D. L.	6.6 ± 0.5	23.5 ± 1.1
R. F.	7.7 ± 0.6	32.6 ± 1.4
S. G.	6.2 ± 0.5	30.6 ± 2.0
B. M.	7.6 ± 0.7	9.1 ± 0.6
H. C.	8.0 ± 0.7	12.7 ± 1.1
S. C.	10.2 ± 0.8	13.8 ± 1.4
G. S.	7.1 ± 0.5	57.1 ± 4.7
R. J.	6.7 ± 0.6	32.5 ± 2.6

* Sera tested were the same as those studied by the bactericidal test (Table V).

streptococci in fresh human blood (Table V). In control experiments (data not shown) the growth of type 6 streptococci was not inhibited in the presence of the immune sera in the fresh blood test mixtures. Uninhibited growth of both type 24 and type 6 streptococci in plasma and immune sera alone confirmed that inhibition of growth required the presence of phagocytic cells. The same sera were also tested by the long-chain test (25) for type-specific anti-M antibody. All sera positive for bactericidal antibodies produced strongly positive type-specific long-chain reactions (Table VI).

For tests of the protective effect of the human M24 antisera, mice were injected intraperitoneally with 0.2 ml of human preimmune or immune sera. 1 d later, the mice were challenged by the same route with various doses of homologous type 24 streptococci or heterologous type 6 streptococci. Of the 12 mice that received preimmune serum and then were challenged with type 24 streptococci, 11 died (Table VII).

TABLE VII

Mouse Protection by Sera of Vaccinees Immunized with pep M24-Alum

Mice injected with human serum from:	No. deaths/No. challenged with:	
	Type 6 strep	Type 24 strep
Unimmunized	7/12	11/12
Vaccinee No. 1	6/12	1/12
Vaccinee No. 2	7/12	1/12

TABLE VIII

Non-Type-specific Immune Response to Injections of pep M24 in Normal Human Volunteers as Measured by CF Test with Crude M30 Antigens *

Vaccinees	Preimmune (serum titer)	Postimmune (serum titer)
D. L.	1:10	<1:10
R. F.	<1:10	<1:10
S. G.	1:20	1:20
B. M.	<1:10	<1:10
H. C.	<1:10	<1:10
S. C.	1:40	1:80
G. S.	1:20	1:20
R. J.	1:10	<1:10

In contrast, only one of each of the groups of 12 mice injected with human immune sera succumbed to type 24 infections. This compared to six or seven deaths in each of the groups of 12 mice injected with preimmune or immune sera and then challenged with type 6 streptococci. These results indicated that vaccination with the pep M24 vaccine induced the formation of type-specific bactericidal, long-chain forming, and protective antibodies in man.

Lack of Cross-Reactivity of the Vaccinees' Sera with Non-Type-specific M-associated Antigens or with Human Heart Tissue. To determine whether or not the vaccinated subjects developed antibody rises to the non-type-specific, M-associated antigens (14), the sera were tested by a microcomplement-fixation test, using an acid-extracted type 30 M protein as antigen. Previous studies (14, 28) have shown that the M30 preparation is rich in antigenic determinants common to M protein preparations from strains of many M types. None of the 12 pep M24 vaccinees developed significant antibody titer rises to the type 30 preparation (Table VIII). Finally, heart-reactive antibodies were not detected in immune sera of any of the twelve vaccinees by immunofluorescence techniques.

Discussion

In this investigation, the immune response to pep M24 was measured by a wide variety of assays which are designed to detect respectively (*a*) type-specific humoral antibodies (opsonocytophagic, long chain, and mouse protection); (*b*) total humoral antibodies (complement fixation [CF] and ELISA); (*c*) cellular immunity (delayed skin test); and (*d*) heart tissue cross-reactive antibodies (immunofluorescence). From these studies, it appears that type-specific primary immune responses in man to the alum-precipitated pep M24 antigen can be stimulated in most individuals with doses that are well-tolerated and that are within conventionally acceptable limits for other

vaccines in common use. This result is gratifying, if not surprising, considering past experience with immunization in man with other partially-purified preparations of M protein (1). Lancefield's early studies (32) in animals comparing the antigenicity of acid-extracted M protein with that of whole heat-killed streptococci suggested that purification of the M antigen was associated with diminished immunogenicity. Indeed, the M antigen was first suspected as being haptenic until its antigenicity was confirmed by its administration to animals in larger doses (33). Most investigators have been disappointed with their inability to achieve a high percentage of primary immune responses in man with the doses of vaccine to which their regimens were confined (34–41). For example, Schmidt (34) was only able to achieve a primary immune response in 2 of 24 children who received a total dose of 300 μg of alum-precipitated type 19 acid-extracted M protein. In studies by Fox and associates (41), acid-extracted and alum-precipitated preparations were injected in relatively small doses (90 μg per dose for three monthly doses) in 19 adults, 5 of which developed opsonic antibody. Because of these reports and our own previous experience (31), we expected that limited peptic digestion and purification of the M protein to the extent of producing a structurally defined molecule containing the M determinant might indeed have resulted in a further loss of immunogenicity. Our findings provide evidence for a stronger and more consistent immune response than we anticipated. Perhaps the reason for the relatively high frequency of our primary responses to pep M24 is the high concentration of specific determinant per weight in our pep M preparation.

The human adult host is naturally hyperimmune to a wide variety of streptococcal protein antigens and most human sera contain antibodies reactive with common non-type-specific M (NTSM) determinants that have not been removable from previous M protein preparations (7–11). Immunization with M protein preparations containing even small amounts of NTSM should, therefore, readily boost such antibody titers. Previous studies have, in fact, shown that pharyngeal infections with group A strains of virulent M types, and particularly those producing rheumatic fever, are associated with high titers of complement-fixing antibodies to NTSM (8, 27). The M protein vaccines used by Fox and associates (41) regularly produced increased levels of passive hemagglutinating antibodies to M proteins, but these antibodies were not type-specific and were therefore probably directed against NTSM. The homogeneity of the immune response to the type-specific determinant in the pep M24 vaccinees is suggested by the absence of increases in complement-fixing antibodies to NTSM in any of these individuals. That only 1 of the 12 individuals immunized with pep M24 showed a significant increase in antibodies directed against pep M6 in the ELISA supports this interpretation. Further studies of the applicability of the ELISA to the measurement of the type-specific determinants of M proteins prepared by the pepsin-extraction method are in progress.[2] If this test can be employed with pep M preparations to measure, reliably and exclusively, type-specific M determinants, the problems of measuring type-specific antibodies by biological tests dependent upon live cultures of virulent streptococci and special donor serum characteristics may be obviated.

The results of the skin test, perhaps the most sensitive indicator of antigenic

[2] Dale, J., I. Ofek, and E. H. Beachey. Enzyme-linked immunosorbent assay (ELISA) of the type specific immune response in humans vaccinated with streptococcal M protein. Manuscript submitted for publication.

homogeneity, also support the type-specificity of the immune response of the vaccinees. 11 of 12 vaccinees developed delayed cutaneous hypersensitivity (DCH) to pep M24 which were mostly of the Jones-Mote type (42). 4 of the 12 vaccinees did, however develop cross-reacting skin tests to pep M6. Alternatively, both preparations might have been contaminated with minute amounts of NTSM that were not detected by any of the other methods for measuring humoral immunity that we employed. On the whole, however, these skin tests represent the most convincing demonstration of type-specific DCH to M protein preparations of which we are aware.

A preliminary study of the pep M24 molecule for homologies with other proteins of known amino acid sequences has been undertaken using a computer search (30). The antigen appears to be unique and related only distantly to other proteins recorded in the program. Whether or not homologies between pep M proteins of different serotypes will be revealed by similar analysis must await further sequencing of the amino acids in such preparations.[3] A comparison of pep M24 and pep M6 by these methods is in progress (J. Seyer and E. H. Beachey. Manuscript in preparation.).

The studies reported here may move us closer to the capability of immunizing man against infection with specific M types of group A streptococci. There is, thus far, no evidence that antibodies to the type-specific M determinant itself are related to the pathogenesis of rheumatic fever. A polyvalent vaccine to structurally defined type-specific M determinants with or without appropriate adjuvants may be useful in certain targeted populations. A recent review of the literature reveals that the number of streptococcal M types that have been clearly associated with rheumatic fever in reports of well-defined outbreaks of group A streptococcal pharyngitis is surprisingly few, perhaps a dozen or less (43). Certain types such as M5 have been particularly frequently associated with rheumatic fever. The so-called skin types causing streptococcal pyoderma do not appear to cause rheumatic fever (44). A polyvalent vaccine against rheumatic fever might, therefore, be confined to a relevant pool of M types found in selected populations. Moreover, a certain targeted population at high risk for rheumatic recurrences, such as young individuals with rheumatic heart disease (45), might benefit by protection against known rheumatogenic M types (46). The absence of any trace of heart-reactive antigens in our preparations and the relative antigenic homogeneity of our preparations may encourage eventual vaccine trials in such selected populations.

Summary

We tested the ability of pepsin-extracted, highly purified M protein to induce type-specific immunity in experimental animals and humans. M protein was prepared from limited peptic digests of whole group A type 24 streptococci and was purified to chemical homogeneity as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, quantitative amino acid analysis, and Edman degradation. For vaccination, the lyophilized M24 protein preparation (pep M24) was precipitated in aluminum hydroxide. When injected into laboratory animals, alum-precipitated pep M24 produced type-specific protective antibodies and was free of non-type-specific immu-

[3] A comparison of the amino terminal segments of pep M24 and a similarly purified polypeptide of type 5 M protein (pep M5) has revealed identical amino acid residues at positions 3, 15, 18, 21, 23, 24, and 27 (J. E. Seyer and E. H. Beachey. Manuscript in preparation.). The significance of these homologies with respect to cross-reactive and protective immunity is now being further investigated.

noreactivity. In man, skin tests with 1-μg doses of pep M24 were negative in all 37 adults tested. 12 adult human volunteers received two–four subcutaneous injections of 100–200 μg of alum-precipitated pep M24 at intervals of at least 2 wk. The immune response to pep M24 was measured by a variety of assays designed to detect (*a*) type-specific humoral antibodies (opsonophagocytic, long chain, and mouse protection tests); (*b*) total humoral antibodies (complement fixation and enzyme-linked immunosorbent assay); (*c*) cellular immunity (skin tests); and (*d*) heart cross-reactive antibodies (immunofluorescence). Type-specific opsonic antibodies developed in 10 of the 12 vaccinees, and positive delayed-type skin tests developed in 11. Immune sera from two of the vaccinees were effective in mouse-protection tests against challenge with M24 but not M6 streptococci. None of the volunteers developed heart-reactive antibodies or antibodies to non-type-specific M protein antigens. Alum-precipitated pep M24 was well-tolerated in man, and no serious local or systemic reactions were observed. Thus, pep M24 induces type-specific, protective antibodies in doses that are well-tolerated in man.

The authors thank Edna Chiang, Robert Curtis, Daniel Monroe, and Kent Williams for expert technical assistance, Dr. Jerome Seyer for performing amino acid analysis, Dr. James Dale for performing the ELISA, Dr. Michael Alkan, who participated in the early part of these studies, and Susan White for secretarial assistance.

Received for publication 2 July 1979.

References

1. Stollerman, G. H. 1975. Rheumatic Fever and Streptococcal Infection. Grune & Stratton, Inc., New York. 101.
2. Kaplan, M. H. 1963. Immunologic relation of streptococcal and tissue antigens. I. Properties of an antigen in certain strains of streptococci exhibiting an immunologic cross-reaction with human heart tissue. *J. Immunol.* **90:**595.
3. Zabriskie, J. B., and E. H. Freimer. 1966. An immunological relationship between the group A streptococcus and mammalian muscle. *J. Exp. Med.* **124:**661.
4. van deRijn, I., J. B. Zabriskie, and M. McCarty. 1977. Group A streptococcal antigens cross-reactive with myocardium: purification of heart-reactive antibody and isolation and characterization of the streptococcal antigen. *J. Exp. Med.* **146:**579.
5. Lancefield, R. C. 1962. Current knowledge of type-specific M antigens of group A streptococci. *J. Immunol.* **89:**307.
6. Beachey, E. H., H. Alberti, and G. H. Stollerman. 1969. Delayed hypersensitivity to purified streptococcal M protein in guinea pigs and in man. *J. Immunol.* **102:**42.
7. Beachey, E. H., and G. H. Stollerman. 1971. Toxic effects of streptococcal M protein on platelets and polymorphonuclear leukocytes in human blood. *J. Exp. Med.* **134:**351.
8. Widdowson, J. P., W. R. Maxted, and A. M. Pinney. 1971. An M-associated protein antigen (MAP) of group A streptococci. *J. Hyg.* **69:**553.
9. Beachey, E. H., and G. H. Stollerman. 1973. Mediation of cytotoxic effects of streptococcal M protein by non-type specific antibody in normal human sera. *J. Clin. Invest.* **52:**2563.
10. Vosti, K. L. 1975. Characterization of nontype specific antigen(s) associated with group A type 12 M protein. *Infect. Immun.* **11:**1300.
11. Fischetti, V. A. 1977. Streptococcal M protein extracted by nonionic detergent. II. Analysis of the antibody response to the multiple antigenic determinants of the M protein molecule. *J. Exp. Med.* **146:**1108.
12. D'Alessandri, R., G. Plotkin, R. M. Kluge, M. K. Wittner, E. N. Fox, A. Dorfman, and R.

H. Waldman. 1978. Protective studies with group A streptococcal M protein vaccine. III. Challenge of volunteers after systemic or intranasal immunization with type 3 or type 12 group A streptococcus. *J. Infect. Dis.* **138:**712.

13. Beachey, E. H., G. L. Campbell, and I. Ofek. 1974. Peptic digestion of streptococcal M protein. II. Extraction of M antigen of group A streptococci with pepsin. *Infect. Immun.* **9:** 891.
14. Beachey, E. H., G. H. Stollerman, E. Y. Chiang, T. M. Chiang, J. M. Seyer, and A. H. Kang. 1977. Purification and properties of M protein extracted from group A streptococci with pepsin: covalent structure of the amino terminal region of type 24 M antigen. *J. Exp. Med.* **145:**1469.
15. Beachey, E. H., J. M. Seyer, and A. H. Kang. Studies of the primary structure of streptococcal M protein antigens. *In* Streptococcal Diseases and the Immune Response. J. B. Zabriskie and S. E. Read, editors. Academic Press, Inc., New York. In press.
16. Beachey, E. H., J. M. Seyer, and A. H. Kang. 1978. Repeating covalent structure of streptococcal M protein. *Proc. Natl. Acad. Sci. U. S. A.* **75:**3163.
17. Lancefield, R. C., and G. E. Perlmann. 1952. Preparation and properties of type-specific antigen isolated from group A, type 1 hemolytic streptococcus. *J. Exp. Med.* **96:**71.
18. Beachey, E. H., E. Y. Chiang, J. M. Seyer, A. H. Kang, T. M. Chiang, and G. H. Stollerman. 1977. Separation of type specific M protein from toxic cross reactive antigens of group A streptococci. *Trans. Assoc. Am. Physicians.* **90:**390.
19. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations in dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244:**4406.
20. Kang, A. H. 1972. Studies on the location of intermolecular cross links in collagen. *Biochemistry.* **11:**1828.
21. Chase, M. W. 1967. Production of antiserum, *In* Methods in Immunology and Immunochemistry, Vol. I. C. A. Williams and M. W. Chase, editors. Academic Press, Inc., New York. 201.
22. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265.
23. U. S. Public Health Service. 1965. Regulations for Biological Products, Title 42, Part 73, General Safety. 14.
24. Cunningham, M. W., and E. H. Beachey. 1975. Immunochemical properties of streptococcal M protein purified by isoelectric focusing. *J. Immunol.* **115:**1002.
25. Stollerman, G. H., A. C. Siegel, and E. E. Johnson. 1959. Evaluation of the "long chain reaction" as a means for detecting type-specific antibody to group A streptococci in human sera. *J. Exp. Med.* **110:**887.
26. Engvall, E., and P. Perlmann. 1972. Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme labeled anti-immunoglobulin in antigen coated tubes. *J. Immunol.* **109:**129.
27. Russell, H., R. R. Facklam, and L. R. Edwards. 1976. Enzyme-linked immunosorbent assay for streptococcal M protein antibodies. *J. Clin. Microbiol.* **3:**501.
28. Beachey, E. H., I. Ofek, and A. L. Bisno. 1973. Studies of the antibodies to nontype specific antigens associated with streptococcal M protein in rheumatic fever. *J. Immunol.* **111:**1361.
29. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.* **27:**493.
30. Dayhoff, M. O., editor. 1978. Atlas of Protein Sequence and Structure. Vol. 5, Suppl. 2. National Biomedical Research Foundation, Wash., D. C. 1.
31. Stollerman, G. H. 1967. Prospects for a vaccine against group A streptococci: the problem of the immunology of M proteins. *Arthritis Rheum.* **10:**245.
32. Lancefield, R. C. 1928. The antigenic complex of *Streptococcus hemolyticus*. II. Chemical and immunological properties of the protein fractions. *J. Exp. Med.* **47:**469.

33. Hirst, G. K., and R. C. Lancefield. 1939. Antigenic properties of the type specific substance derived from group A hemolytic streptococci. *J. Exp. Med.* **69**:425.
34. Schmidt, W. C. 1960. Type-specific antibody formation in man following injection of streptococcal M protein. *J. Infect. Dis.* **106**:250.
35. 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 protein from group A, type 14 streptococci. *J. Lab. Clin. Med.* **61**:459.
36. Potter, E. V., G. H. Stollerman, and A. C. Siegel. 1962. Recall of type-specific antibodies in man by injections of streptococcal cell walls. *J. Clin. Invest.* **41**:301.
37. Massell, B. F., J. G. Michael, J. Amezcua, and M. Siner. 1968. Secondary and apparent primary antibody responses after group A streptococcal vaccination of 21 children. *Appl. Microbiol.* **16**:509.
38. 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. Exp. Med.* **124**:1135.
39. Fox, E. N., L. M. Pachman, M. K. Wittner, and A. Dorfman. 1969. Primary immunization of infants and children with group A streptococcal M protein. *J. Infect. Dis.* **120**:598.
40. Wittner, M. K., and E. N. Fox. 1971. Micro complement fixation assay for type specific group A streptococcal antibody. *Infect. Immun.* **4**:441.
41. Fox, E. N., R. H. Waldman, M. K. Wittner, A. A. Mauceri, and A. Dorfman. 1973. Protective study with group A streptococcal M protein vaccine. Infectivity challenge of human volunteers. *J. Clin. Invest.* **52**:1885.
42. Askenase, P. W., and J. E. Atwood. 1976. Basophils in tuberculin and "Jones-Mote" delayed reaction in humans. *J. Clin. Invest.* **58**:1145.
43. Bisno, A. L. The concept of rheumatogenic and non-rheumatogenic group A streptococci. *In* Streptococcal Infections and the Immune Response. J. B. Zabriskie and S. E. Read, editors. Academic Press, Inc., New York. In press.
44. Wannamaker, L. W. 1973. The chain that links the heart to the throat. *Circulation.* **48**:9.
45. Taranta, A. 1967. Factors influencing recurrent rheumatic fever. *Annu. Rev. Med.* **18**:159.
46. Stollerman, G. H. 1975. The relative rheumatogenicity of strains of group A streptococci. *Mod. Concepts Cardiovasc. Dis.* **44**:35.