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Heterogeneity of Group A Type-Specific Antibodies

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Extracting type 6 group A streptococci with dilute nitrous acid releases an antigen that exhibits type-specific precipitating properties with anti-M6 serum but fails to neutralize the type-specific bactericidal reaction or to absorb the bactericidal antibody from anti-M6 serum. These studies suggest that the precipitating and antiphagocytic properties of the M protein are attributable to different antigenic determinants. The antigenic determinant responsible for bactericidal antibody is more sensitive to the deleterious effect of nitrous acid than is the moiety responsible for the precipitin reaction. Use of the indirect fluorescent technique with anti-M sera absorbed with lyophilized nitrous acid extracts permits the possible differentiation of the two M-protein determinants in situ on the surface of streptococcal cells.

The M-proteins of group A streptococci are a group of immunologically distinct substances that form the basis of classifying these organisms into serological types of clinical and epidemiological significance (20). They are important because they stimulate the production of type-specific antibodies that neutralize the antiphagocytic property of M antigen. These bactericidal antibodies may confer long-lasting immunity to the particular type causing the streptococcal infection.

Among the several techniques for detecting M-protein antibodies in immunized rabbits, the precipitin test (28) and the bactericidal test (19) are the most commonly used. In general, there is a good correlation between the serotype specificity as detected by the precipitin test and the bactericidal test (17, 19). Consequently most investigators have surmised that the same antigen participates in both the precipitin and the bactericidal system, and furthermore, that the same population of antibodies is involved in each system. Because of this generally good correlation between the occurrence and the specificity of the antigens or antigenic determinants active in the bactericidal and the Lancefield extract precipitin systems, the latter, more easily performed reaction is commonly used to detect the M protein in epidemiological surveys (6, 23).

Laboratories immunizing large numbers of rabbits have observed rabbit immune sera that are precipitating but not bactericidal, and vice versa. In addition, certain instances of M serotypes exhibiting serological cross-reactions with one kind of test system but not with the other have been reported (11, 14, 29, 30).

Recently, several groups of investigators have suggested that the antigenic determinants for the opsonic and precipitating antibody activities are different. Beachey and his colleagues (E. H. Beachey, P. Belew, and G. H. Stollerman, Bacteriol. Proc., p. 76-77, 1970) examined M protein by Sephadex chromatography and electrophoresis on acrylamide gel. They described several fractions with precipitating activity, but only one of these fractions could neutralize the opsonic system. Ofek et al. (22) also described two fractions of partially purified M protein derived from sonically disrupted cells. Both fractions had precipitating activity, but only one could induce opsonic antibodies. Cunningham and Beachey (5) described a partially purified M-protein fraction prepared by alkaline extraction and isoelectric focusing that could inhibit opsonization of homologous type-specific streptococci by M antibody but did not induce opsonic antibodies in rabbits or precipitate type-specific antiserum. Russell and Facklam (25) prepared, from guanidine-extracted cells, partially purified M-protein fractions with different biological activities. Two fractions precipitated with whole antiserum, but only one of these two fractions could neutralize opsonic activity of the same antiserum. The fraction with opsonic neutralizing capacity elicited opsonic antibodies in rabbits, but the fractions that did not have opsonic neutralizing capacity did not. Wittner (31) described experiments demonstrating the removal of the M12 opsonic activity but only partial removal of the M12 precipitating activity of M12 antiserum by purified heterologous M proteins. Fischetti et al. (10), using M proteins

extracted by nonionic detergent, reported that absorption of type-specific opsonic serum with small-molecular-weight type-specific M-protein molecules resulted in the removal of the ability of the serum to precipitate without affecting the opsonic power. All of these results indicate that the two biological activities are to some degree separate. However, Beachey and his colleagues (1, 4, 5), using a mild peptic digestion, were able to purify M protein from M24 group A streptococci that possessed both opsonic inhibitory and precipitating activities.

The studies reported here describe another new method of extracting M protein, with dilute nitrous acid. The findings support the view that the antigenic determinant for the antiphagocytic property of the M protein and that for the precipitating reaction are separate and distinct, eliciting two different antibody populations.

MATERIALS AND METHODS

Strains. Serotypes M1 (T1/195/2), M2 (T2/44/RB4/19), M5 (T5B/126/3), M6 (S43/192/1), M14 (T14/46/7), and M24 (C98/105/2) were obtained from Rockefeller University, New York, N.Y. All strains were serologically confirmed as group A, and specific type reactions were demonstrated with absorbed homologous M antisera.

Antisera. Specific M typing antisera for types 1, 2, 5, 6, 14, and 24 were obtained from Rockefeller University (courtesy of R. C. Lancefield). Additional M6 antisera were obtained from the Center for Disease Control, Atlanta, Ga., and prepared in our own laboratory.

Dilute nitrous acid extraction techniques. The nitrous acid extraction procedure used for this study differed from that previously reported in that a more dilute final concentration of the extracting agent was employed (7). The washed cells of a 500-ml overnight Todd-Hewitt broth culture were suspended in 0.5 ml of saline for each gram of wet cells. A 100-μl volume of 4 M sodium nitrite was added to the saline suspension of cells and mixed well, and then 50 μl of glacial acetic acid was added to the mixture. After the suspension was thoroughly mixed, it was allowed to stand at room temperature for 15 min. The pH was raised initially with 10 N NaOH and then with 0.2 N NaOH to a fine adjustment of pH 7.4. The supernatant that was obtained after the suspension was centrifuged at 3,000 rpm is the dilute nitrous acid extract used in these experiments.

All experiments with dilute nitrous acid and other extracts were made in triplicate, and the results of the various procedures were read and recorded "blindly."

Bactericidal test. The bactericidal test used was a modification of the original Lancefield technique (19). A 0.1-ml volume of an overnight Todd-Hewitt broth culture of the test strain was transferred to a 5-ml volume of Todd-Hewitt broth. The culture was incubated for 4 h in a 37°C water bath, after which it was diluted 10^{-4}, and this dilution was diluted further to 1:4, 1:16, and 1:64. Saline broth (5% Todd-Hewitt broth plus 0.85% NaCl, vol/vol) was used as diluent. A 0.025-ml portion of each dilution was removed to determine counts of colony-forming units (CFU) by plating in blood agar, and 0.025 ml of each of the four culture dilutions (10^{-4}, 1:4, 1:16, 1:64) was added to tubes (13 by 100 mm) containing 0.025 ml of the test serum. The same amounts of the four culture dilutions were also added to tubes containing 0.025 ml of normal rabbit serum. These tubes served as controls and indicated the growth capacity of the different dilutions of the test strain. Freshly collected heparinized (5 U/ml) human blood (0.3 ml) was then added to each tube. The tubes were tightly sealed with rubber stoppers and rotated end-over-end (8 rpm) for 3 h at 37°C. At the end of the 3 h, 0.025 ml of the contents of each tube was removed and placed in 2.5 ml of saline broth in the bottom of a petri dish (15 by 100 mm). Ten milliliters of melted agar containing 5% rabbit blood was added. After mixing, the inoculated agars were allowed to solidify and were incubated overnight at 37°C. The surviving streptococci form beta-hemolytic colonies in the blood agar plate. The colonies were counted and are expressed as CFU in this report.

Bactericidal inhibition tests. Two methods were used to determine the ability of dilute nitrous acid extracts to inhibit the opsonic activity of M-antisera. In the first, 0.025 ml of extract was added directly to the bactericidal system. The second method was similar to that described by Beachey et al. (3) and involved absorbing M-antiserum with dilute nitrous acid extract before its addition to the bactericidal system. In both cases, the bactericidal test was then performed as described above without further modification.

Absorption of sera with dilute nitrous acid extracts. Dilute nitrous acid extracts were dialyzed against distilled water for 16 to 18 h at 4°C. The dialysis tubing had a cut-off of 6,000 to 8,000 daltons (Spectrum Medical Industries, Los Angeles, Calif.). The dialyzed dilute nitrous acid extract was then lyophilized to a powder. The lyophilized, dialyzed, dilute nitrous acid extract (2 to 4 mg) was used to absorb type 6-specific antiserum (0.2 ml) for 1 h at 37°C. The mixture was clarified by centrifugation (3,000 rpm for 20 min). The supernatant contained the absorbed M6 antiserum used in these studies.

Other techniques. Semipurified M protein was prepared according to the techniques described by Lancefield and Perlmann (21). Some of the semipurified M-protein extracts were concentrated by dialysis and lyophilization in a manner similar to that described for concentrating the dilute nitrous acid extracts. Electron micrographs were prepared according to the procedures described by Swanson et al. (27). Culture (21), cell wall preparation (26), digestion by trypsin (18), hot-HCl extraction (16), precipitin reaction in capillaries and in gel immunodiffusion (16, 24), immunoelectrophoresis (13), and fluorescent microscopy (15) were performed according to previously described techniques. Fluorescein-labeled goat anti-rabbit and anti-gamma globulin was obtained from Farbwerke, Hoechst AG, Frankfurt, W. Germany.

RESULTS

In a previous report, the successful use of nitrous acid for polysaccharide extraction and

grouping of beta-hemolytic streptococci was described (7). M protein could not be detected in these extracts by either the bactericidal or the precipitin test. Earlier, Swanson et al. reported electron microscopic evidence that the streptococcal cells exposed to nitrous acid retain their fimbriae-like structures and react with ferritin-labeled specific immune sera; they concluded that nitrous acid does not extract M protein (27).

Subsequent studies in our laboratory have shown, however, that by using a more dilute final concentration of nitrous acid than the one used for polysaccharide extraction, a supernatant can be obtained which gives a specific precipitation with homologous anti-M serum in capillaries and a line of identity in double immunodiffusion with a hot-HCl (Lancefield) extract from the same serotype (Fig. 1). Identical results were also obtained with extracts from streptococcal cell walls.

After digestion with trypsin, dilute nitrous acid extracts did not give a precipitin reaction with homologous anti-M serum. Dilute nitrous acid extracts also failed to precipitate when they were extracted from trypsinized streptococcal whole cells and cell wall preparations.

Up to three repeated extractions with the dilute HNO_2 yielded similar precipitating extracts from the same M6 streptococcal culture. Moreover, cells extracted with nitrous acid, then subsequently extracted with hot HCl, yielded extracts that precipitated the absorbed M6 serum (Fig. 1). These results indicate that a single extraction with HNO_2 does not deplete the cells of the precipitating moiety.

The specificity of the precipitin reaction obtained in capillaries and in an immunodiffusion system with dilute nitrous acid extracts was tested. In these studies three heterologous prototype strains, M1, M5, and M24, were used. Nitrous acid extracts from whole cells or cell walls of these heterologous strains did not precipitate with anti-M6 immune sera and did not absorb their precipitin antibodies.

Although dilute nitrous acid extracts (those obtained from single or repeated extractions) specifically absorbed out the precipitating antibodies of homologous immune sera, they did not absorb out the bactericidal antibodies of the homologous immune sera. Table 1 shows the results of a series of bactericidal neutralization and absorption experiments. The first line gives the number of streptococcal CFU included in each dilution. System A shows the control bactericidal system. The M6 strain was inhibited in the presence of homologous antiserum. The results in system B were obtained when undialyzed M6 dilute nitrous acid extracts were added to this homologous bactericidal system. The undialyzed extract appeared to have no effect on the bactericidal system with homologous antiserum. However, a nonspecific effect was demonstrated on the heterologous system containing M1 antiserum apparently because of the toxicity of the undialyzed nitrous acid extract on the streptococcal organism. (The results for the control are shown in system A, Table 2.) As results for system C show, dialyzed dilute nitrous acid extracts of M6 strains did not appear to influence either the homologous or the heterologous system. Thus, the dialyzed extract showed no evidence of toxicity for either the streptococci or the leukocytes. As with addition to the bactericidal system, preadsorption of the anti-M6 serum with the dilute nitrous acid M6 extract had no effect on the homologous bactericidal system (system D).

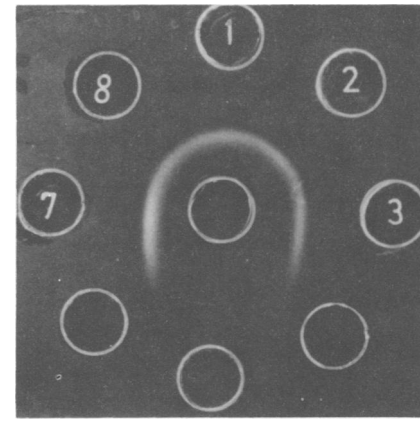

FIG. 1. *Double gel diffusion reaction in agar gel with group A streptococcal extracts and M6 antiserum. Center well, Absorbed M6 serum. Peripheral wells: 1, M6 Lancefield HCl extract; 2 and 3, M6 nitrous acid extract; 7 and 8, M6 Lancefield HCl extract from cells previously extracted with dilute nitrous acid.*

Data shown in Table 2 confirm the nontoxicity of the nitrous acid extract to the leukocytes in a heterologous bactericidal system. In system A (control), M1 antiserum inhibited the growth of the M1 strain. System B shows that the nitrous acid extract after dialysis was not toxic to the leukocytes in the test and allowed the phagocytosis of M1 cells in the presence of opsonic antibody.

Precipitin reactions and neutralization of bactericidal activities of dilute nitrous acid extracts are summarized in Table 3. Nitrous acid extracts of whole cells and cell walls precipitated with but did not neutralize the bactericidal activity of homologous M antiserum. Trypsin destroyed the precipitin activity of the nitrous acid extract. Hot-HCl extracts, crude or semipurified, precip-

TABLE 1. *Effects on the M6 bactericidal system of adding dilute nitrous acid extracts and absorbing M antiserum with these extracts*

System	Components of tests		CFU per dilution of M6 streptococci[a]			
	Antiserum[b]	Extract	10^{-4} (406)	1:4 (128)	1:16 (27)	1:64 (13)
A	NRS	None	962	808	22	4
	M6	None	2	0	0	0
B	M6	M6 NA[c] (before dialysis)	0	0	0	0
	M1	M6 NA (before dialysis)	59	29	0	0
C	M6	M6 NA (after dialysis)	15	0	0	0
	M1	M6 NA (after dialysis)	TNTC[d]	834	624	176
D	M6, absorbed with M6 NA	None	6	3	0	0

[a] Actual counts; multiply by 14 to obtain CFU per tube. Parentheses indicate number of CFU inoculated.
[b] Center for Disease Control M6 antiserum. Similar results were obtained with Rockefeller University and Cairo M6 antisera. NRS, Normal rabbit serum.
[c] NA, Dilute nitrous acid extract.
[d] TNTC, Too numerous to count.

TABLE 2. *Effects on the M1 bactericidal system of adding dilute nitrous acid extracts*

System	Components of tests		CFU per dilution of M1 streptococci[a]			
	Antiserum[b]	Extract	10^{-4} (1,044)	1:4 (320)	1:16 (67)	1:64 (27)
A	NRS	None	TNTC[c]	TNTC	TNTC	294
	M1	None	8	13	3	0
B	M1	M6 NA[d] (after dialysis)	56	6	2	0

[a] Actual counts; multiply by 14 to obtain CFU per tube. Parentheses indicate number of CFU inoculated.
[b] Center for Disease Control antiserum. Similar results were obtained with Rockefeller University and Cairo antisera. NRS, Normal rabbit serum.
[c] TNTC, Too numerous to count.
[d] NA, Dilute nitrous acid extract.

itated with homologous antisera. The ability of the hot-HCl extracts to neutralize the bactericidal activity of homologous antisera cannot be evaluated because those extracts were toxic to leukocytes. Nitrous acid treatment of the semipurified hot-HCl extracts or of whole cells before hot-HCl extraction did not affect the precipitating activity of the hot-HCl extracts and removed the toxic effect to leukocytes.

To determine whether these observations were a peculiarity of a particular antiserum, we examined three anti-M6 rabbit sera from three different laboratories. These sera had been absorbed with a heterologous strain in the usual manner for preparing type-specific antisera and are hereafter referred to as "complete" sera. The sera were absorbed with the dilute dialyzed lyophilized HNO_2 extracts. These sera were no longer capable of precipitating with homologous hot-HCl or nitrous acid extracts. The bactericidal property for the specific serotype was, however, retained. Sera absorbed in this manner will be referred to as "bactericidal" sera. Lyophogel concentration (fivefold) of the bactericidal sera did not elicit any precipitation with homologous M antisera and did not affect the bactericidal properties of the sera.

Further studies were directed toward determining whether the two antisera would react differently with native or altered M protein on the surface of streptococcal cells. The indirect fluorescent antibody technique (with fluorescein-labeled anti-rabbit globulin) was used with both the complete anti-M6 rabbit serum and the corresponding bactericidal serum (absorbed with homologous dilute nitrous acid extract).

The prototype M6 strains used in these experiments grew well in human blood lacking antibody for this type, were phagocytized in the presence of homologous M6 antiserum in bactericidal tests, and had Lancefield extracts that precipitated with homologous M6 antiserum. When examined by the indirect fluorescent antibody technique, these strains reacted with both

TABLE 3. *Reactions of nitrous acid and Lancefield extracts from M6 Rockefeller strain S43/192/1 with homologous immune rabbit sera*[a]

Extracts	Precipitation in capillaries and immunodiffusion	Neutralization of bactericidal activity
Dilute HNO_2 extract of whole cells	Yes	No
Dilute HNO_2 extract of cell wall	Yes	No
Dilute HNO_2 extract after digestion with trypsin	No	No
Hot-HCl extracts of whole cells	Yes	Toxic effect
Hot-HCl extracts (semipurified) plus dilute HNO_2 and dialysis	Yes	Toxic effect
Hot-HCl extract of cells previously exposed to dilute HNO_2[b]	Yes	No

[a] Center for Disease Control serum; same results were obtained with Rockefeller and Cairo rabbit sera.
[b] The cells were washed three times in physiological saline after exposure to nitrous acid and before hot-HCl extraction.

TABLE 4. *Reaction in the indirect fluorescein antibody test of M6 streptococcal preparations with specific M6 antiserum, absorbed and nonabsorbed with dilute nitrous acid extracts*

Strains	Fluorescein staining[a]	
	With complete antiserum 1/620[b]	With bactericidal M6 antiserum 1/620[c]
M6 cells (untreated) growing in human blood	+4	+4
M6 cells extracted by nitrous acid	+4	0
M6 cells extracted by HCl	+3	+3
M1 cells, M14 cells, M2 cells	±1	0

[a] Indirect technique with fluorescein-labeled anti-rabbit gamma globulins prepared in goats (Hoechst).
[b] Serum is precipitating and bactericidal.
[c] Serum is not precipitating, only bactericidal.

the complete and the bactericidal antisera (Table 4). After being treated with nitrous acid, the cell sediments reacted only with the complete antiserum. Evidently, the nitrous acid treatment altered or destroyed the antiphagocytic antigen. Streptococcal cells from the original culture, after Lancefield hot-HCl extraction, continued to react with both the complete and the bactericidal antisera. This indicated that not all the precipitating and antiphagocytic antigen(s) was removed by the Lancefield extraction technique. Cells from group A streptococci of M types 1, 2, and 14 did not stain with either antiserum.

Electron microscopy of streptococcal cells extracted with the dilute HNO_2 showed that the fimbriae-like structures were retained while the precipitating moiety was being extracted with dilute nitrous acid (Fig. 2).

DISCUSSION

The reported experiments with the M6 immunological system strongly suggest that the specific anti-M6 rabbit sera contain at least two antibody populations that can be identified and differentiated by specific absorption with an antigenic determinant present in the dilute HNO_2 extract. The antigen in the nitrous acid extract precipitates specifically in capillaries, is sensitive to trypsin, and gives a complete line of identity with the standard Lancefield hot-HCl extract in an immunodiffusion system, but it does not absorb out the bactericidal antibodies.

These experiments, along with those performed with fluorescent staining, suggest that nitrous acid results in an alteration of M protein but gave us no evidence as to whether the nitrous acid procedure actually extracts the antiphagocytic determinant.

Our results appear to support the findings of Fischetti et al. (10) and Russell and Facklam (25). These investigators presented evidence that the antigenic moieties for precipitin and opsonic activities were different. In fact, Fischetti has reported that the molecular configuration of the M-protein molecule may play a more important role than primary sequence in determining the antiphagocytic properties (8, 9). Recently, however, Beachey and his colleagues (1, 4) demonstrated that a protein moiety that contained the antigenic determinants eliciting both precipitating and opsonic antibodies could be extracted and purified. Beachey and his colleagues (2) recently reported that each of seven peptides obtained from purified M protein possessed the type-specific determinant that inhibits opsonic activity. These results appear to contradict the results reported here and by Fischetti. However, we began our experiments, as did Fischetti, with type 6 group A streptococci, whereas Beachey used type 24 for his studies. In addition, Beachey did not present evidence that both serological activities were present in the antibody. He did present evidence that the antigen evoking the antibodies were on the same protein molecule.

Our data indicate that the precipitin test does not correlate with the opsonic (bactericidal) test,

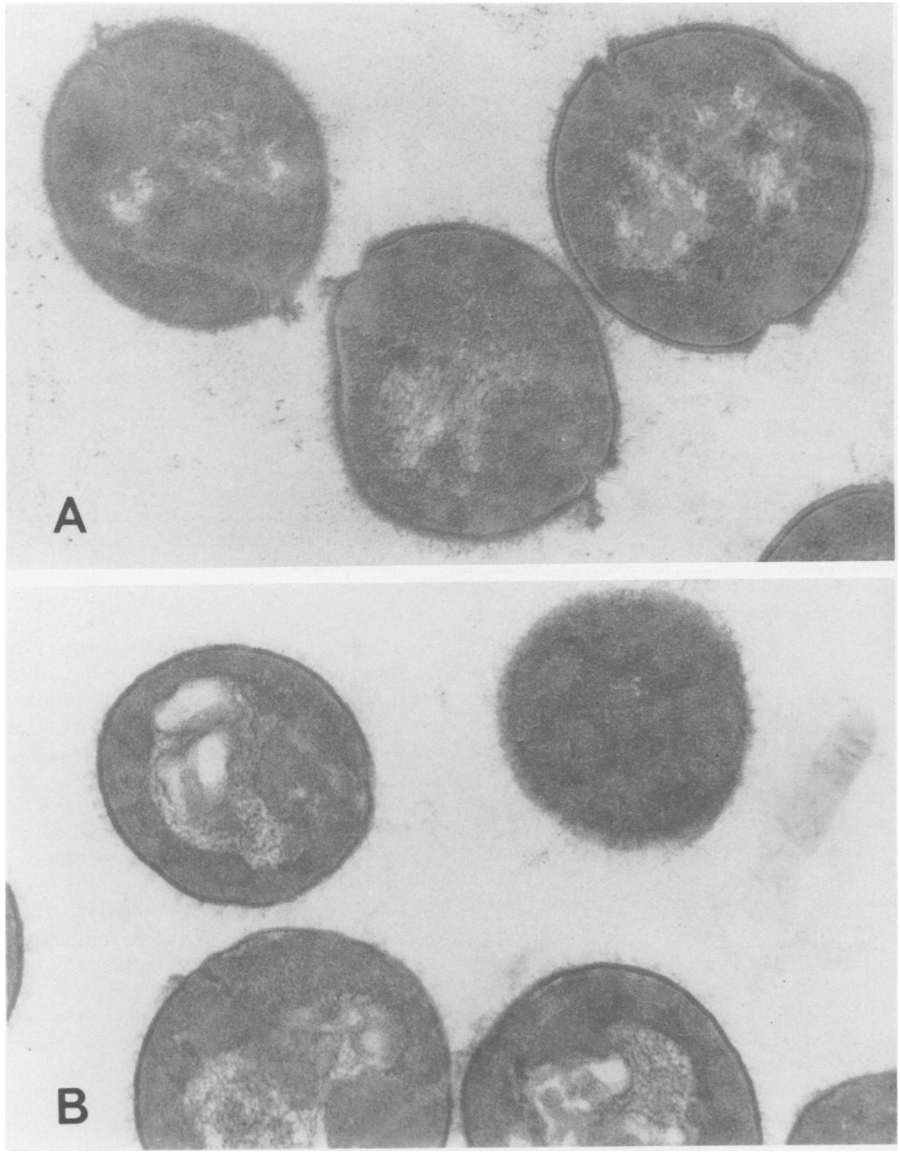

FIG. 2. *Electron micrograph of group A M6 streptococci (magnification, ×64,000). (A) Before extraction; (B) after treatment with nitrous acid.*

at least for the system we have described. There may be type-to-type differences in group A streptococcal strains and variations in extraction techniques that could also lead to different results and conclusions.

These findings do not necessarily contradict the results reported by Swanson et al. (27). Neither the hot-HCl nor the dilute nitrous acid extraction procedure depleted completely the streptococcal cell wall of the precipitating moiety. Thus, the ferritin-labeled globulins reacting with the fimbriae-like structures in the electron micrographs of Swanson and collaborators could have been reacting with the precipitating determinant of the M protein remaining in the cells.

One possible practical extension of these studies would be to use the specific bactericidal antibody to screen strains by the indirect fluorescent antibody technique to identify those that might produce bactericidal antibody in rabbits.

Further work is needed to determine whether the different antigenic determinants of the M proteins are carried by a single molecule or by

different molecules and, perhaps more significantly, whether the M proteins of freshly isolated group A streptococcal strains always carry both antigenic determinants.

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LITERATURE CITED

1. **Beachey, E. H., G. L. Campbell, and I. Ofek.** 1974. Peptic digestion of streptococcal M-protein. II. Extraction of M antigen from group A streptococci with pepsin. Infect. Immun. **9**:891–896.
2. **Beachey, E. H., J. M. Sayer, and A. H. Kang.** 1978. Repeating covalent structure of streptococcal M-protein. Proc. Natl. Acad. Sci. U.S.A. **75**:3163–3167.
3. **Beachey, E. H., and G. H. Stollerman.** 1971. Toxic effects of streptococcal M-proteins on platelets and polymorphonuclear leukocytes in human blood. J. Exp. Med. **134**:351–365.
4. **Beachey, E. H., G. H. Stollerman, E. Y. Chaing, T. M. Chaing, J. B. Sayer, and A. H. Kang.** 1977. Purification and properties of M-proteins extracted from group A streptococci with pepsin: covalent structure of the amino acid terminal region of the type 24 M-protein. J. Exp. Med. **145**:1469–1483.
5. **Cunningham, M., and E. H. Beachey.** 1975. Immunochemical properties of streptococcal M-protein purified by isoelectric focusing. J. Immunol. **115**:1002–1015.
6. **El Kholy, A. M., A. H. Sorour, H. B. Houser, L. W. Wannamaker, M. Robbins, J. M. Poitras, and R. M. Krause.** 1973. A three-year prospective study of streptococcal infections in a population of rural Eygptian school children. J. Med. Microbiol. **6**:101–110.
7. **El Kholy, A. M., L. W. Wannamaker, and R. M. Krause.** 1974. Simplified extraction procedure for serological grouping of beta-hemolytic streptococci. Appl. Microbiol. **28**:836–839.
8. **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**:1108–1123.
9. **Fischetti, V. A.** 1978. Streptococcal M-protein extracted by nonionic detergent. III. Correlation between immunological cross-reactions and structural similarities with implications for antiphagocytosis. J. Exp. Med. **147**: 1771–1778.
10. **Fischetti, V. A., E. C. Gotschlich, G. Siviglia, and J. B. Zabriskie.** 1976. Streptococcal M-protein extracted by nonionic detergent. I. Properties of the antiphagocytic and type-specific molecules. J. Exp. Med. **144**:32–53.
11. **Fox, E. N., and M. K. Whittner.** 1968. Antigenicity of the M-proteins of group A hemolytic streptococci. IV. Cross reactivity between serotypes. J. Immunol. **100**: 39–45.
12. **Griffith, F.** 1934. The serological classification of Streptococcus pyogenes. J. Hyg. Cam. **34**:542–584.
13. **Halbert, S. P.** 1971. The analysis of streptococcal infections. VI. Immunoelectrophoretic observations on extra-cellular antigens detectable with human antibodies. J. Exp. Med. **113**:1013–1028.
14. **Harrell, W. K., H. Ashworth, and R. E. Davis II.** 1971. Cross-protective antigens of group A streptococci types 3 and 31 and types 46 and 51. Infect. Immun. **4**:79–84.
15. **Karakawa, W. W., E. K. Borman, and C. R. McFarland.** 1964. Typing of group A streptococci by immunofluorescence. I. Preparation and properties of type 1 fluorescein-labeled antibody. J. Bacteriol. **87**:1377–1382.
16. **Lancefield, R. C.** 1933. A serological differentiation of human and other groups of hemolytic streptococci. J. Exp. Med. **57**:571–595.
17. **Lancefield, R. C.** 1940–1941. Specific relationship of cell composition to biological activity of hemolytic streptococci. Harvey Lect. Ser. **36**:251–290.
18. **Lancefield, R. C.** 1943. Studies on the antigenic composition of group A hemolytic streptococci. I. Effects of proteolytic enzymes on streptococcal cells. J. Exp. Med. **78**:465–476.
19. **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. Exp. Med. **106**:525–544.
20. **Lancefield, R. C.** 1962. Current knowledge of type-specific "M" antigens of group A streptococci. J. Immunol. **89**:307–313.
21. **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. Exp. Med. **96**:71–82.
22. **Ofek, I., S. Bergner-Rabinowitz, and A. M. Davies.** 1969. Opsonic capacity of type specific streptococcal antibodies. Israel J. Med. Sci. **5**:293–296.
23. **Parker, M. T.** 1967. International Survey of the distribution of serotypes of Streptococcus pyogenes (Group A streptococci). Bull. W.H.O. **37**:513–527.
24. **Rotta, J., R. M. Krause, R. C. Lancefield, W. Everly, and H. Lackland.** 1971. New approaches for the laboratory recognition of M types of group A streptococci. J. Exp. Med. **134**:1289–1315.
25. **Russell, H., and R. R. Facklam.** 1975. Guanidine extraction of streptococcal M-protein. Infect. Immun. **12**: 679–686.
26. **Salton, M. R. J., and R. W. Horne.** 1951. Studies of the bacterial cell wall. II. Methods of preparation and some properties of cell walls. Biochem. Biophys. Acta **7**:177–197.
27. **Swanson, J., R. C. Hsu, and E. C. Gotschlich.** 1969. Electron microscopic studies of streptococci. I. M antigen. J. Exp. Med. **130**:1063–1091.
28. **Swift, H. R., A. T. Wilson, and R. C. Lancefield.** 1943. Typing group A hemolytic streptococci by M precipitin reactions in capillary pipettes. J. Exp. Med. **78**:127–133.
29. **Wiley, G. G., and P. N. Bruno.** 1968. Cross-reactions among Group A streptococci. I. Precipitin and bactericidal cross reactions among types 33, 41, 43, 52 and Ross. J. Exp. Med. **128**:959–968.
30. **Wiley, G. G., and A. T. Wilson.** 1961. The occurrence of two M antigens in certain group A streptococci related to type 14. J. Exp. Med. **113**:451–465.
31. **Wittner, M. K.** 1976. Antigenicity of the M-proteins of group A hemolytic streptococci: further evidence for shared determinants among serotypes. Infect. Immun. **13**:634–642.