INFECTION AND IMMUNITY, June 1975, p. 1300-1306

Vol. 11, No. 6
Printed in U.S.A.

Characterization of a Non-Type-Specific Antigen(s) Associated with Group A Streptococcal Type 12 M Protein

KENNETH L. VOSTI

Division of Infectious Diseases, Stanford University School of Medicine, Stanford, California 94305

Received for publication 6 February 1975

The sharing of one and possibly two or more non-type-specific antigens by most strains of groups A, C, and G streptococci is described. With the exception of a single strain of *Proteus mirabilis*, this antigen(s) was not found among strains of groups B and D streptococci, coagulase-positive staphylococci, *Escherichia coli*, *Pseudomonas* sp., and *Salmonella* sp. The non-type-specific antigen(s) could not be separated from M protein by fractionation with various saturations of ammonium sulfate or by column chromatography with calcium hydroxylapatite even though the latter method allowed the recovery of a fraction of M protein which was free of the cross-reactive antigen(s). The resistance of this non-type-specific antigen(s) to hot acid extraction and its sensitivity to treatment with trypsin differentiate it from the T and R antigens of group A streptococci, both of which are trypsin resistant. Co-precipitation of both type- and non-type-specific antigens occurred with type-specific antiserum and suggested that the type- and non-type-specific antigens represent either different, covalently bonded antigenic determinants on the same protein or different proteins noncovalently linked together.

The antigenic structure of the cell wall of group A hemolytic streptococci is complex (14, 17, 19, 21). Two antigens, group A carbohydrate and M protein, have received particular attention primarily because of the importance of the grouping antigen to the primary serologic classification of hemolytic streptococci and of the M proteins to the subtyping and virulence of strains of group A streptococci. In addition, two other cell wall proteins, T and R, have been characterized (14, 21). Antisera to the T antigens, although not sharing the same type specificity as that directed toward the M antigens, have provided a useful additional method for subclassifying strains of group A streptococci. Only M proteins stimulated bactericidal antibodies and protective immunity (14, 17, 19, 21).

For many years the presence of additional non-type-specific proteins has interfered with attempts to purify M proteins (15, 16, 25) and to develop a serologic assay for type-specific antibody (6, 11, 27). Renewed interest in these antigens has been stimulated recently by attempts to purify M proteins (1, 3-5, 8-10, 12, 26, 28) and by the possibility that certain M-associated, non-type-specific antigens cross-react with tissue antigens and therefore may be involved in the pathogenesis of poststreptococcal diseases (2, 13, 20, 22, 29).

The present study assesses the occurrence of a non-type-specific antigen(s) found in a semipurified preparation of type 12 M protein among strains of other M types of group A streptococci, groups B, C, D and G streptococci, and other gram-positive and -negative bacteria. Characterization of the non-type-specific antigen(s) and attempts to separate it from M protein are described.

MATERIALS AND METHODS

Bacterial strains. The strains were grown from frozen stock cultures maintained in this laboratory for over 10 years or from fresh isolates from clinical materials obtained from the diagnostic microbiology laboratory. *Pseudomonas* sp. were identified by colonial morphology and the following biochemical reactions: alkaline reaction throughout butt and slant on triple sugar iron agar, reduction of nitrates, oxidase positive, utilization of glucose oxidatively, and failure to utilize maltose or 10% lactose. The *Enterobacteriaceae* were identified by the classification scheme of Edwards and Ewing (7). *Staphylococcus aureus* was differentiated from *Staphylcoccus epidermidis* by a positive 6- and 24-h coagulase test. Group D *Streptococcus* of the enterococcal variety was identified by growth in litmus milk and 6.5% NaCl in brain heart infusion broth. The other beta-hemolytic streptococci were subclassified by the method of Lancefield (17), using grouping and typing antisera obtained from the Bureau of Laboratories Division of the Center for Disease Control, Atlanta, Ga. The group A, M type 12, T type 12 (Shaw) streptococcus was originally isolated from the excised tonsil of an asymptomatic child and has served as the

source of type 12 M protein used in the indirect hemagglutination system for the assay of type 12-specific antibody (27).

Preparation of antigens. The individual strains were grown in brain heart infusion broth (Difco). Flasks containing 800 ml of culture material were inoculated with 0.5 ml of a seed culture and incubated for 18 h at 37 C. The organisms were collected by centrifugation; 3 ml of packed cells was suspended in 7 ml of 0.2 N HCl and heated in boiling water for 10 min. The supernatant fluids were neutralized with 0.2 N NaOH, and after overnight refrigeration at 4 C any precipitates which formed were removed by centrifugation.

The protein precipitated from the clear supernatant fluid by 33 to 60% saturation with $(NH_4)_2SO_4$ was dissolved in 10 ml of phosphate-buffered saline (PBS), pH 7.2, and precipitated a second time. The second 33 to 60% precipitate was dissolved in PBS (pH 7.2) and dialyzed overnight against the same buffer. The semipurified antigen used for sensitization of tanned erythrocytes was dissolved in PBS (pH 6.4) and dialyzed overnight against the same buffer. Most of the final semipurified antigens contained between 300 to 1,000 μg of protein nitrogen per ml as determined by the method of Lowry et al. (18), using bovine albumin (Armour Pharmaceutical Co.) as the reference standard.

Hemagglutination inhibition technique. Human, group O, Rhesus-negative erythrocytes were tanned and sensitized as described previously (27). Starting with 1:10, 12 master dilutions were prepared with one of three human sera. Each of these sera was known to contain levels of antibody of 1:320 to 1:2,560 for the cross-reactive antigen(s) present on cells sensitized with the semipurified type 12 (Shaw) M protein. The presence of a similar cross-reactive antigen(s) in the test antigen was assayed by incubating 0.05 ml of the test antigen with each serum dilution at room temperature for 1 h prior to the addition of the sensitized erythrocytes. Cross-reactive antigen(s) was considered to be present if the test antigen caused a decrease in titer of three tubes or greater over that observed in the control dilution to which only 0.05 ml of PBS (pH 7.2) had been added.

Double diffusion in agar. The method of Ouchterlony (23) was used with 1% Noble agar in 0.9% NaCl and 0.075 M phosphate buffer (pH 7.3) with 1:10,000 merthiolate as a preservative. The plates were developed with either absorbed rabbit sera which contained group A- or type 12-specific (3/17/69, lot 7) precipitating antibodies (Bureau of Laboratories, Center for Disease Control, Atlanta, Ga.) or unabsorbed rabbit antisera which contained cross-reactive precipitating antibodies (prepared by repeated intravenous injections of whole cells killed by heating at 56 C for 30 min of group A streptococci of M types 1, 3, 6 or 17).

Analytic disc (polyacrylamide gel) electrophoresis. Methods were used as described previously (12). For analysis of acrylamide gels by double diffusion, the gels were quick frozen in acetone-dry ice slushes and sliced into 5-mm sections with a razor blade. The sections were placed directly into the antigen wells of the double diffusion plate.

Hydroxylapatite column chromatography. Separation of the semipurified (second 33 to 60% precipitate) type 12 M protein was performed as described previously (12).

Enzymatic digestion. One milligram of protein in PBS (pH 7.1) of semipurified (33 to 60% precipitate) type 12 M protein obtained from the Shaw strain was incubated with 20 μg of trypsin, 2× crystallized (Nutritional Biochemicals Corp., Cleveland, Ohio), for 50 min at 36 C, in a shaking water bath. The mixture was chilled to 0 C, and antigen activity was assessed by double diffusion in agar.

Co-precipitation experiment. Volumes of 0.1 ml of a semipurified type 12 M protein (33 to 60% precipitate) were mixed with either 0.2 ml of normal rabbit serum in a small test tube or 0.2 ml of rabbit antiserum containing antibodies for M12 protein but not for the cross-reactive antigen(s). The specificity of this antiserum (anti-"b") has been demonstrated previously (26). The mixtures in the two tubes were incubated at 5 C for 18 h and centrifuged at 18,000 × *g*, and the clear supernatant fluid was assayed by double diffusion in agar.

RESULTS

Cross-reactive antigen(s). The distribution of the non-type-specific antigen(s) found in the semipurified preparation of type 12 M protein was assessed by hemagglutination inhibition among similar antigen extracts prepared from a variety of different bacteria. As seen in Table 1, the non-type-specific antigen(s) was not restricted to strains of group A hemolytic streptococci. It was also present in extracts of four of five strains of group C and five of six strains of

TABLE 1. *Occurrence among various bacteria of a non-type-specific antigen(s) present in a semipurified preparation of type 12 M protein*

Source extract	No. positive[a]/ no. of strains
Group A streptococci	7/9
M type 1	1/1
M type 3	1/1
M type 6	2/2
M type 12	2/2
M type 17	1/1
M type 22	0/1
M type 44	0/1
Group B streptococci	0/5
Group C streptococci	4/5
Group G streptococci	5/6
Group D streptococci, enterococci	0/6
Coagulase-positive staphylococci	0/6
Escherichia coli	0/5
Proteus mirabilis	1/5
Pseudomonas sp.	0/5
Salmonella sp.	0/6

[a] A three-tube (eightfold) or greater decrease of control titer.

group G streptococci but in none of those prepared from strains of group B and D streptococci or coagulase-positive staphylococci. With the exception of one strain of *Proteus mirabilis*, extracts of strains of gram-negative bacteria failed to contain the cross-reactive antigen(s). As little as 5 μg of protein nitrogen from some extracts of strains of groups C and G inhibited the test system five tubes or more whereas 40 to 70 μg of protein nitrogen from some of the non-reactive extracts from other bacteria failed to cause any inhibition. Fifteen of the 17 positive extracts produced a decrease in titer of five tubes or greater.

Characterization of the sensitizing antigen. The semipurified type 12 M protein (33 to 60% precipitate) which was used for sensitization of the tanned erythrocytes formed a single strong and a single weak precipitin band with absorbed type 12-specific antiserum but not with antiserum directed against the group A carbohydrate (Fig. 1). Although this semipurified antigen failed to react with grouping antisera, it formed one to two precipitin bands with unabsorbed antisera prepared against whole cells of M types 1, 6, and 17. The line formed closest to the antigen well with the type 12 antiserum gave a reaction of identity with one of the lines formed with heterologous antisera.

The heterogeneity of the semipurified antigen was further reflected by separation of the proteins in polyacrylamide gels. Some 13 to 15 bands were identified on stained gels. Approximately 25, 30, 30, and 15% of the stainable protein was found, respectively, between R_f's of 0 to 25, 25 to 50, 50 to 75, and 75 to 100% of the distance migrated by the bromophenol blue marker. Type-specific antigen was found in each of the 5-mm cuts taken from the start of the spacer gel to the bromophenol blue marker (Fig. 2). This contrasted with the presence of non-type-specific antigens only in the first two or three gel slices.

Purification of the cross-reactive antigen. The fractions derived from the crude acid extract of the type 12 Shaw strain by precipitation of protein with various saturations of ammonium sulfate were screened for both M-specific and non-type-specific antigens. No fraction was identified which contained either antigen free from the other.

Further separation of the cross-reactive and type-specific antigens found in semipurified type 12 protein (33 to 60% precipitate) was attempted by column chromatography with hydroxylapatite. As seen in Fig. 3, three peaks were recovered. Type 12-specific antigen was present only in the "a" and "b" fractions. Although the "b" fraction contained only type 12-specific antigen, the "a" fraction contained both type-specific and cross-reactive antigens (Fig. 4). No fraction contained only cross-reactive antigen(s) devoid of type-specific antigen.

Enzymatic digestion. The semipurified type 12 antigen was subjected to enzymatic digestion with trypsin. Both the type 12 specific and the non-type-specific antigens were destroyed by incubation with the enzyme for 50 min at 37 C in a shaking water bath.

Co-precipitation. A further attempt was made to separate the type and non-type-specific antigens by precipitation of the type-specific antigen with type-specific antiserum as described above. The type-specific and non-

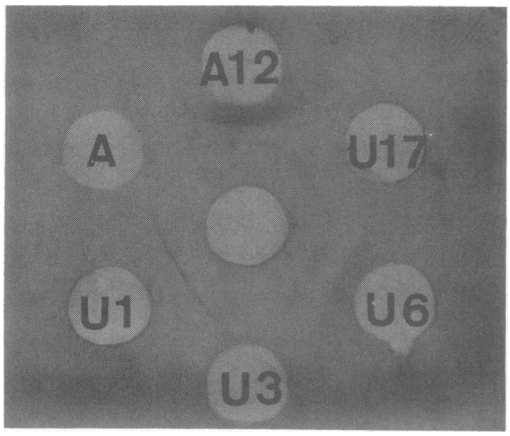

FIG. 1. *Ouchterlony reactions of semipurified type 12 M protein with absorbed type 12, unabsorbed types 1, 3, 6, and 17, and group A antisera.*

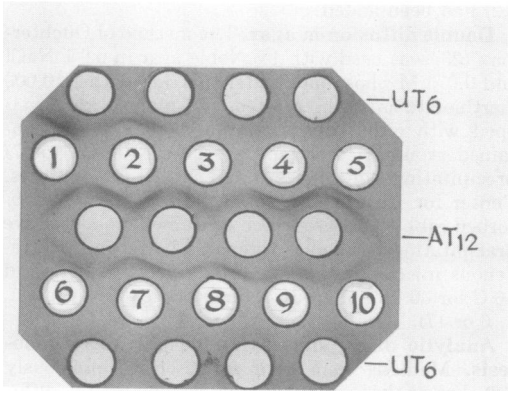

FIG. 2. *Ouchterlony reactions of acrylamide gel fractions of semipurified type 12 M protein with absorbed type 12 and unabsorbed type 6 antisera.*

type-specific antigens were co-precipitated by type-specific antibody (Fig. 5).

DISCUSSION

The cross-reactive antigen(s) detected by inhibition of hemagglutination and by precipitation in gels in the present and previous (12, 17, 26) studies presumably is the same or quite similar to that identified by the complement fixation test (28), cytotoxicity (3), and skin reactivity (24). The present study with inhibition of hemagglutination confirms the previous observation with the complement fixation test that the non-type-specific antigen(s) is not limited to strains of group A streptococci but occurs in a significant proportion of strains of groups C and G. Widdowson et al. (28) did not find this antigen(s) among representative strains of group B and F streptococci. We also failed to find the cross-reactive antigen in concentrated acid extracts of strains of group B and, in addition, among similarly prepared extracts of strains of group D enterococci, coagulase-positive staphylococci, and several species of gram-negative bacteria. The presence of the cross-reactive antigen(s) in a single strain of *P. mirabilis* in the present study is unexplained but suggests that its distribution may not be limited to gram-positive bacteria.

The exact number of different non-type-specific antigens is not known. Neither the complement fixation test utilized by Widdowson et al. (28) nor the inhibition of hemagglutination test used in the present study allowed an accurate assessment of the complexity of the cross-reactions. Although the antigen used to sensitize the erthrocytes was shown to contain at least two cross-reactive antigens by precipitin reactions in gels, the presence of antibodies in the human sera used in our experiments and in those of others for more than one of the antigens was not known.

The cross-reactive antigens described in this and other studies (28) survived extraction from whole cells or cell walls by boiling for 10 min in 0.2 N HCl but were destroyed by extraction

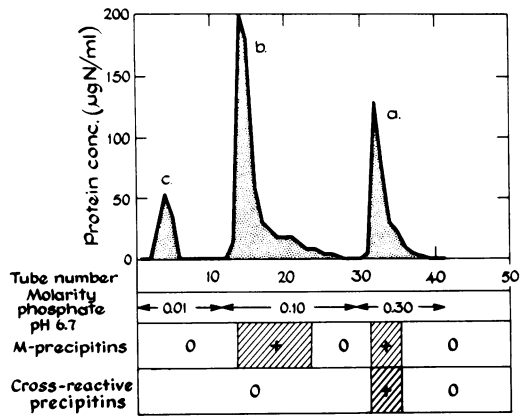

FIG. 3. *Further separation of semipurified type 12 M protein by stepwise elution from a column of calcium hydroxylapatite.*

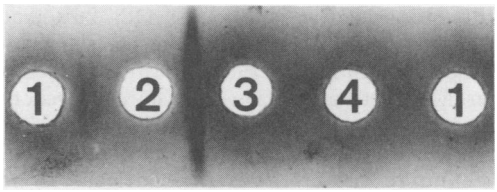

FIG. 5. *Co-precipitation of M-specific and cross-reactive antigens by type-specific antibody. (1) Unabsorbed whole cell group A, M type 6 antiserum; (3) M type 12-specific antiserum; (2) final supernatant fluid of the mixture of semipurified M12 protein plus normal rabbit serum or (4) final supernatant fluid of the mixture of semipurified M12 protein plus M type 12-specific antiserum.*

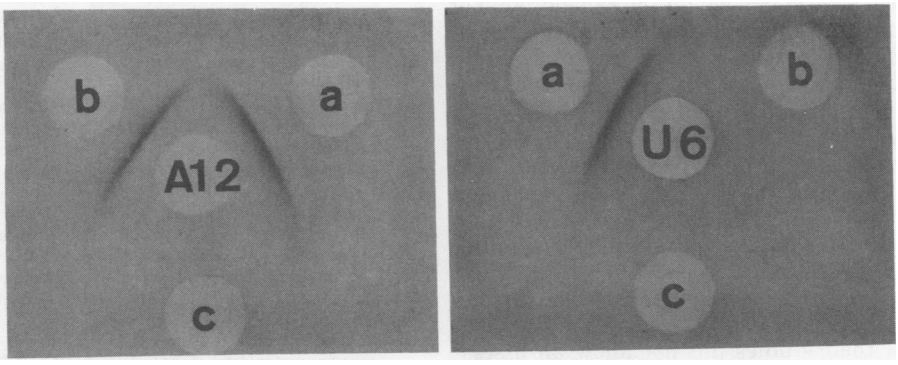

FIG. 4. *Ouchterlony reactions of hydroxylapatite fractions a, b, and c with absorbed type 12 (A12) and unabsorbed type 6 (U6) antisera.*

with formamide or by incubation with trypsin, pepsin, or streptococcal proteinase. The resistance of this cross-reactive antigen to acid extraction and sensitivity to trypsin differentiate it from the T and R antigens (19). The separation of the cross-reactive antigen(s) from M protein was not achieved in the present study by fractionation with varying degrees of saturation with ammonium sulfate or by column chromatography with hydroxylapatite. This experience is similar to that of Widdowson et al. (28) with column chromatography with carboxymethylcellulose since increased recovery of cross-reactive antigens was associated with increased recovery of M protein. They also failed to detect cross-reactive antigen in an M negative strain of group A *Streptococcus*. These observations led these authors to refer to the cross-reactive antigen(s) as M-associated proteins. Unlike their experience with carboxymethyl-cellulose, column chromatography with hydroxylapatite allowed the recovery of a fraction of M protein which was free of cross-reactive antigen(s). Thus, it is clear that a fraction of M protein released by acid extraction is free of cross-reactive antigen(s) and therefore that its activity and stability does not depend on the presence of the cross-reactive antigen(s) (12, 26). Additional support for this observation is provided by Cunningham and Beachey (5), who derived an M reactive fraction free of cross-reactive antigen(s) by partial degradation with pepsin. Further understanding of the relationship of M protein to the cross-reactive antigens is provided by the co-precipitation experiment in the present study. The co-precipitation of both type- and non-type-specific antigens by antibody directed against only the type-specific antigens by antibody directed against only the type-specific antigen suggests that the cross-reactive antigen(s) and type-specific antigen represent either different antigenic determinants covalently bonded on the same protein or different antigenic proteins that are noncovalently linked together.

The relationship of the cross-reactive antigen(s) to the shared tissue antigen(s) described by others (13, 20, 22, 29) was not assessed in the present study. However, Widdowson et al. (28) were able to remove antibody for M-associated proteins from high-titered human sera by absorption with whole cells of streptococci, protoplast membranes of a type 49 M positive strain, and human heart and skeletal muscle. As discussed earlier, the complement fixation test used in their studies did not allow an assessment of the actual number of antigens involved. It seems probable that not all of the cross-reactive antigens found among strains of streptococci share antigenic determinants with tissue antigens. The wide distribution of at least one of these cross-reactive antigens among strains of groups C and G, which are generally not considered to be associated with rheumatic fever or glomerulonephritis, seems consistent with this probability if the shared antigens are related to the pathogenesis of the late complications of group A streptococcal infections. The separation and purification of the individual cross-reactive antigens will most likely be required to satisfactorily resolve many of the unanswered questions concerning their complexity, biological significance, and relationships to other streptococcal antigens.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-06964 from the National Institutes of Allergy and Infectious Diseases.

LITERATURE CITED

1. 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**:42-52.
2. Beachey, E. H., I. Oftek, and A. L. Bisno. 1973. Studies of antibodies to non-type-specific antigens associated with streptococcal M protein in the sera of patients with rheumatic fever. J. Immunol. **111**:1361-1366.
3. Beachey, E. H., and G. H. Stollerman. 1972. The common antigen(s) of streptococcal M protein vaccines causing hyperimmune reactions in man. Trans. Assoc. Am. Physicians **85**:212-220.
4. Beachey, E. H., and G. H. Stollerman. 1973. Mediation of cytotoxic effects of streptococcal M protein by non-type-specific antibody in human sera. J. Clin. Invest. **52**:2563-2570.
5. Cunningham, M. W., and E. H. Beachey. 1974. Peptic digestion of streptococcal M protein. I. Effect of digestion at suboptimal pH upon the biological and immunological properties of purified M protein extracts. Infect. Immun. **9**:244-248.
6. Denny, F. W., Jr., and L. J. Thomas. 1953. The demonstration of type specific streptococcal antibody by a hemagglutination technique employing tannic acid. J. Clin. Invest. **32**:1085-1093.
7. Edwards, P. R., and W. H. Ewing. 1972. Identification of *Enterobacteriaceae*. Burgess Publishing Co., Minneapolis, Minn.
8. Fox, E. N., and M. K. Wittner. 1965. The multiple molecular structure of the M proteins of group A streptococci. Proc. Natl. Acad. Sci. U.S.A. **54**:1118-1125.
9. Fox, E. N., and M. K. Wittner. 1969. New observations on the structure and antigenicity of the M proteins of the group A streptococcus. Immunochemistry **6**:11-24.
10. Havlicek, J., J. E. Alouf, and M. Raynaud. 1969. Heterogeneite antigenique de la proteine M de *Streptococcus pyogenes* type 24. Ann. Inst. Pasteur Paris **117**:745-755.
11. Holmes, M. 1958. Type specific antibodies to *Streptococcus pyogenes*. Aust. J. Exp. Biol. Med. Sci. **36**:395-404.
12. Johnson, R. H., and K. L. Vosti. 1968. The purification of

two fragments of M protein from a strain of group A, type 12 streptococcus. J. Immunol. **101**:381-391.
13. Kaplan, M. H. 1964. Immunologic cross-reaction between group A streptococcal cell walls and mammalian tissues—possible relationship to induction of autoimmunity in rheumatic fever, p. 169-184. *In* J. W. Uhr (ed.), The streptococcus, rheumatic fever and glomerulonephritis. The Williams and Wilkins Co., Baltimore.
14. Krause, R. M. 1972. The streptococcal cell: relationship of structure to function and pathogenesis, p. 5-7. *In* L. W. Wannamaker and J. M. Matsen (ed.), Streptococci and streptococcal diseases. Academic Press, New York.
15. Lancefield, R. C. 1928. The antigenic complex of *Streptococcus hemolyticus*. I. Demonstration of a type-specific substance in extracts of *Streptococcus hemolyticus*. J. Exp. Med. **47**:91-103.
16. Lancefield, R. C. 1928. The antigenic complex of *Streptococcus hemolyticus*. II. Chemical and immunological properties of the protein fractions. J. Exp. Med. **47**:469-480.
17. Lancefield, R. C. 1940-41. Specific relationship of cell composition to biological activity of hemolytic streptococci. Harvey Lect. **36**:251-290.
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. Biol. Chem. **193**:265-275.
19. McCarty, M. 1965. The hemolytic steptococci, p. 356-390. *In* R. J. Dubois and J. G. Hirsch (ed.), Bacterial and mycotic infections of man. J. B. Lippincott Co., Philadelphia.
20. Markowitz, A. S., and C. F. Lange, Jr. 1964. Streptococcal related glomerulonephritis. I. Isolation, immunochemistry and comparative chemistry of soluble fractions from type 12 nephritogenic streptococci and human glomeruli. J. Immunol. **92**:565-575.
21. Maxted, W. R., and J. P. Widdowson. 1972. The protein antigens of group A streptococci, p. 251-266. *In* L. W. Wannamaker and J. M. Matsen (ed.), Streptococci and streptococcal diseases. Academic Press, New York.
22. Nakhla, L. S., and L. E. Glynn. 1967. Studies on the antigen in β-hemolytic streptococci that cross-reacts with an antigen in human myocardium. Immunology **13**:209-218.
23. Ouchterlony, O. 1953. Antigen-antibody reactions in gels. IV. Types of reactions in coordinated systems of diffusion. Acta Pathol. Microbiol. Scand. **32**:231-240.
24. Pachman, L. M., and E. N. Fox. 1970. Cellular and antibody reactions to streptococcal M protein types 1, 3, 6 and 12. J. Immunol. **105**:898-907.
25. Rantz, L. A., and E. Randall. 1947. Antibacterial precipitating antibodies in group A hemolytic streptococcus sore throat. Am. J. Med. **2**:551-567.
26. Vosti, K. L., R. H. Johnson and, M. F. Dillon. 1971. Further characterization of purified fractions of M protein from a strain of group A, type 12 streptococcus. J. Immunol. **107**:104-114.
27. Vosti, K. L., and L. A. Rantz. 1964. The measurement of type and non-type-specific group A hemolytic streptococcal antibody with an hemagglutination technique. J. Immunol. **92**:185-191.
28. Widdowson, J. P., Maxted, W. R., and A. M. Pinney. 1971. An M-associated protein antigen (MAP) of group A streptococci. J. Hyg. **69**:553-564.
29. Zabriskie, J. B., and E. H. Freimer. 1966. An immunologic relationship between the group A streptococcus and mammalian muscle. J. Exp. Med. **124**:661-678.