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Immunochemical Analysis of Intact M Protein Secreted From Cell Wall-Less Streptococci

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M protein is a major virulence factor of group A streptococci, which provides these organisms with protection against phagocytosis in the absence of specific antibody. To gain insight into the nature of the native M-protein molecule, type 12 M protein was isolated and purified from the extracellular supernatants of a group A streptococcal L form and stabilized protoplasts. The intact purified M protein from both sources had a molecular weight of 58,000, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This is in contrast to the 32,000-dalton molecule isolated from the parent type 12 organism by using a nonionic detergent. The purified secretory M protein removed opsonic antibodies from type 12 rabbit immune serum, as demonstrated by a bactericidal assay. Therefore, it appears that either previous nondestructive methods of M-protein isolation have not removed intact M protein from cell walls or part of the molecule is fragmented during its association with cell walls.

The M protein of group A streptococci protects these organisms from phagocytosis in the absence of antibody specific for the antiphagocytic sites on the M-protein molecule. Initially, M protein was extracted for immunochemical studies by using hot acid, as described by Lancefield (15). Other methods, such as alkaline pH (8), guanidine hydrochloride (20), streptococcal phage-associated lysin (5, 7, 11, 13), pepsin (1), and nonionic detergents (6), have also been used to remove M protein from the surfaces of streptococcal cell walls for immunochemical and structural analyses. Of the nondestructive extraction methods, molecular weight determinations using dissociating conditions have been accomplished only with nonionic detergent-extracted M protein (33,000 daltons) (6). Molecular weights have been estimated for alkaline pH-extracted M protein; however, these estimates were made under non-dissociating conditions, resulting in an apparent molecular weight of 116,000 to 180,000. Analyses of the M-protein sequence have indicated that its structure allows for the association of the basic molecule into a larger complex (17). In addition, polyacrylamide gradient gel electrophoresis has revealed that the M-protein molecules have a tendency to aggregate into a larger molecular species (unpublished data).

To gain a better understanding of the native M-protein molecule, we examined the properties of the M protein secreted by organisms lacking cell walls (i.e., L forms and protoplasts) (9, 10, 12, 21). The M-protein molecules isolated from the extracellular products of these organisms were purified and compared with detergent-extracted M protein isolated from the parent of the L form. The M proteins secreted from both the osmotically stabilized protoplasts and the L forms had a molecular weight of 58,000, compared with a molecular weight of 32,000 for the M protein extracted with detergent from the parent. In addition, immunodiffusion revealed that the secreted M-protein molecule contained at least one immunodeterminant not found on the smaller M protein. Finally, we found that the secreted M protein was able to absorb type-specific opsonic antibody from rabbit immune sera.

MATERIALS AND METHODS

Bacteria and media. Streptococcal strain 416 and its L form were obtained from W. R. Maxted, Central Public Health Laboratory, London, England. Strain 416 was grouped and typed as group A type 12, whereas the L form produced only the soluble type M protein. The L form did not produce the group carbohydrate.

Strain 416 was grown in dialyzed Todd-Hewitt broth as previously described (25). The L form was grown for several passages in brain heart infusion (Difco Laboratories, Detroit Mich.) containing 0.3 M sucrose and 10% horse serum. Once the L form was adapted to this medium, the horse serum concentration was reduced to 3%. The L form and protoplasts (see below) were grown in the presence of 0.1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.) and Trasylol (Mobay Chemical Co., New York, N.Y.).

Preparation and growth of protoplasts. Strain 416 was grown in dialyzed Todd-Hewitt broth, and

protoplasts were prepared by using phage-associated lysin in the presence of 30% raffinose as previously described (24), with the following modifications. After what appeared to be 100% conversion of streptococci to protoplasts by phase-contrast microscopy, protoplasts were sedimented by centrifugation at 8,000 × *g* in a Sorvall RC2B centrifuge (Du Pont Instruments, Newtown, Conn.). The pellet was resuspended in the protoplasting solution without enzymes and resedimented as described above. The protoplasts were then suspended in dialyzed Todd-Hewitt broth containing 30% raffinose and incubated at 37°C for 18 h. Next, the protoplasts were removed by centrifugation at 10,000 × *g* for 30 min, and the supernatant was filtered through a 0.22-μm membrane filter (Millipore Corp., Bedford, Mass.). Finally, the filtrate was centrifuged at 100,000 × *g* for 60 min to remove any residual protoplasts that passed through the filter.

Fractionation of M protein secreted from L forms and protoplasts. Throughout these procedures, the protease inhibitors phenylmethylsulfonyl fluoride and Trasylol were present in all solutions at concentrations of 1 mM. Identical procedures were used to purify the two forms of extracellular M protein.

(i) Method 1 (for volumes of less than 1 liter). A type 12 affinity column was prepared by coupling gamma globulin from type 12 absorbed sera to Sepharose 4B with cyanogen bromide by the method of March et al. (18). The growth supernatants isolated as described above were dialyzed directly against 10 mM sodium phosphate buffer (pH 6.0) containing 0.5 M sodium chloride. They were then loaded onto the affinity column (1.5 by 5 cm) at a flow rate of 5 to 10 ml/h, after which the column was run with the loading buffer until the optical density at 280 nm reached base line. The bound extracellular M protein was then eluted at 10 ml/h by using 0.1 M sodium citrate buffer (pH 3.1). Finally, the column was stripped with 3 M ammonium thiocyanate to remove any residual tightly bound M protein.

The fractions containing M protein were identified by double-diffusion analysis, using type-specific antisera. If necessary, as determined by sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis (see below), any contaminated fractions were purified further by carboxymethyl cellulose chromatography, as previously described (6).

(ii) Method 2 (for volumes of more than 1 liter). We found that the affinity technique (method 1) was not efficient for the isolation of extracellular M protein from larger quantities of extracellular fluids. The L-form or protoplast supernatants of larger volumes were first precipitated with 55% saturated ammonium sulfate in order to concentrate the M protein. The precipitate was collected by centrifugation at 10,400 × *g* for 30 min in a Sorvall RC2B centrifuge. Pellets were suspended in 5 mM sodium acetate buffer (pH 5.5) and dialyzed extensively against the same buffer. Any insoluble precipitate was removed by centrifugation at 27,000 × *g* for 10 min, and the supernatant was brought to 12.5% concentration with polyethelene glycol (molecular weight, 6,000) and allowed to stand overnight. The precipitate which formed was sedimented by centrifugation at 27,000 × *g* for 30 min and then solubilized in 5 mM sodium acetate buffer (pH 5.5). This material was then loaded onto a column of carboxymethyl cellulose (1.5 by 15 cm; CM 52; Whatman Ltd., Clifton, N.J.) that was equilibrated with 5 mM sodium acetate buffer (pH 5.5). The column was washed until the optical density at 280 nm reached base line. A linear gradient of 0 to 1 M sodium chloride in equilibration buffer was then used to release the M protein. Fractions were assayed for M protein as described above. The fractions showing immunological reactivity with type-specific antisera were then pooled and dialyzed against 10 mM sodium phosphate buffer (pH 6.5).

The partially purified M protein was then loaded onto a column of hydroxyapatite (1.5 by 15 cm; Bio-Gel HT; Bio-Rad Laboratories, Richmond, Calif.) equilibrated with 10 mM phosphate buffer. A gradient of sodium phosphate (pH 6.5; 10 mM to 0.5 M; 200 ml) was used to elute the M protein from the column. Fractions were analyzed for M protein, and the appropriate fractions were dialyzed against 10 mM sodium phosphate (pH 6.5). The partially purified L-form M protein was then brought to 20% saturation with ammonium sulfate and loaded onto a column of phenyl Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). The M protein was then eluted by using a 20 to 0% ammonium sulfate gradient in 10 mM sodium phosphate buffer (pH 6.5). The serologically active fractions were pooled.

Preparation of detergent-extracted M protein. Cell walls were prepared by the method of Bleiweis et al. (3), using a vibrogen cell mill (RHO Scientific, Commack, N.Y.), and M protein was extracted and purified as described previously (6).

Polyacrylamide slab gel electrophoresis. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis, using a discontinuous tris(hydroxymethyl)aminomethane-hydrochloride buffer system (14). Electrophoresis was performed on a 7 to 15% gradient slab polyacrylamide gel at 25 mA until the indicator dye reached the bottom of the gel.

Standards (bovine serum albumin, ovalbumin, carbonic anhydrase, and cytochrome *c*) were run on all gels for molecular weight determinations. Samples and standards containing 2% sodium dodecyl sulfate, 5% mercaptoethanol, and 10% glycerol were boiled for 2 to 5 min in a water bath before being loaded onto the gel. After electrophoresis, the gels were stained and destained as described previously (24).

Indirect bactericidal assay. Bactericidal assays were performed as described by Lancefield (16). Stationary tubes without serum were used to control the growth of cells in the heparinized blood of the donors. Rotated tubes without serum were run as controls for the active growth of streptococci in the presence of the phagocytes. Runs were considered valid only when both controls exhibited complete ("laked") hemolysis.

Acid extraction. Acid extracts containing M protein were prepared by the method of Lancefield (15). the neutralized supernatant containing M protein was filtered through a 0.22-μm Millipore membrane and then stored at 4°C in the presence of 0.02% sodium azide.

Antisera. Antisera were prepared in rabbits by inoculating them with whole heat-killed streptococcal

vaccines (19). Adsorbed type-specific antisera were prepared as described by Rotta et al. (19); sera were absorbed with heterologous streptococcal M types to remove all cross-reactions with extracts from these types.

New Zealand white rabbits (12 to 16 kg) were each immunized with 50 μg of purified L-form M protein in complete Freund adjuvant. After 3 weeks each rabbit was boosted with 25 μg of purified L-form M protein in incomplete Freund adjuvant; 1 week after the booster dose, the rabbits were bled for sera containing antibodies against the L-form M protein.

Gamma globulin was prepared from rabbit antiserum by chromatography on diethylaminoethyl cellulose, as previously described (25).

RESULTS

Preliminary experiments. Initial experiments were performed to determine whether L forms and protoplasts secreted M protein into the growth medium. The extracellular fluids were concentrated 10-fold and used in double-diffusion studies. As Fig. 1A shows, the extracellular fluids from both the L forms and the type 12 protoplasts reacted with specific type 12 antiserum, indicating the presence of M protein. Acid-extracted M protein and nonionic detergent-extracted M protein from the parent strain showed lines of partial identity with the extracellular M protein, suggesting that additional antigenic determinants were present on the secreted M-protein molecule. Similar results were observed when purified extracellular M protein was substituted for the crude preparations (see below).

Sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis of the purified extracellular M protein. Once it was established immunologically that type-specific M protein was secreted from both L forms and stable protoplasts, larger quantities of extracellular fluids were prepared, and the secreted M proteins were purified as described above. Sodium dodecyl sulfate gel electrophoresis of the purified M proteins secreted from both the L forms and the protoplasts revealed a single polypeptide at the same relative position (Fig. 2). Molecular weight estimates indicated that both extracellular M-protein molecules had a molecular weight of 58,000, compared with detergent-extracted M protein, whose molecular weight was 32,000. Therefore, it appears from these results that either previous methods of M-protein isolation have not removed intact M proteins from the cells or cleavage of the secreted 58,000-dalton molecules occurs, resulting in the insertion of 32,000-dalton molecules into the cell walls.

Gel diffusion experiments. To clarify the nature of the extra determinant(s) present on the secreted M-protein molecule, preparations were analyzed by double diffusion, using anti-M-protein antisera. As noted above, the L-form and protoplast secreted M proteins exhibited lines of identity when analyzed with type 12-specific antisera and showed lines of partial identity when compared with acid-extracted type 12 M protein (Fig. 1A and Table 1). In addition, the M protein isolated by detergent extraction from the parent type 12 strain also exhibited a line of partial identity with the L-form and protoplast M pro-

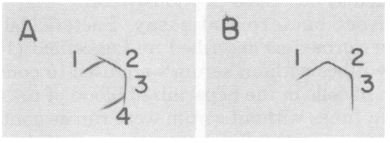

FIG. 1. *Immunodiffusion analysis of extracellular type 12 M-proteins with acid- and detergent-extracted type 12 M proteins. The following preparations were tested in the outer wells. (A) Well 1, Acid-extracted M protein; well 2, L-form M protein; well 3, protoplast M protein; well 4, detergent-extracted M protein. The center well contained type 12-specific antiserum 1. (B) Well 1, Acid-extracted M protein; well 2, L-form M protein; well 3, detergent-extracted M protein. The center well contained type 12-specific antiserum 2.*

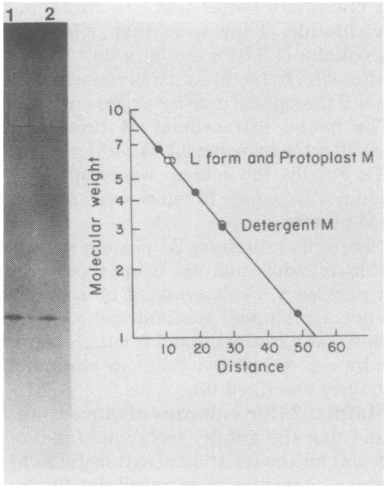

FIG. 2. *Sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis of purified extracellular type 12 M proteins. Samples of purified L-form (lane 1) and protoplast (lane 2) M proteins were loaded onto 7 to 15% sodium dodecyl sulfate-polyacrylamide gradient gels and run as described in the text. Single polypeptides were resolved at the same positions for both extracellular M-protein preparations. Molecular weight estimates were 58,000 for the secretory M protein and 32,000 for detergent-extracted M protein* (○). *The protein standards used* (●) *were bovine serum albumin (molecular weight, 68,000), ovalbumin (43,500), carbonic anhydrase (30,000), and cytochrome (12,300).*

teins. Similar results were obtained when antiserum against the purified L-form M protein was used. However, we found that not all type 12-specific antisera contained antibodies against the extra determinant(s). Figure 1B demonstrates the absence of antibodies against the extra determinant(s), as shown by the reaction of identity with a type 12 antiserum different from the one used in Fig. 1A. Of four type-specific antisera tested, two contained antibodies to the extra determinant(s).

To explore the possibility that the spur observed was the result of a common determinant(s) found in the M proteins of other streptococcal serotypes, the L-form M protein was tested by gel diffusion against unabsorbed antisera prepared against 57 other M-protein types (Table 1). In no case did an unabsorbed serum react with the L-form M protein, suggesting the absence of a common immunodeterminant in the larger M-protein molecule.

Finally, to demonstrate the absence of any group A or group A variant carbohydrate determinant in the L-form and protoplast M-protein preparations, the proteins were reacted against anti-group A and anti-group A variant carbohydrate antisera (Table 1). The results demonstrated that neither of these two carbohydrates was present in the extracellular M-protein preparations, suggesting that the observed spur was not due to the presence of a carbohydrate moiety on the molecule.

Therefore, the gel diffusion data suggest that the spur found as a result of certain type 12 antisera was probably due to a serotype-specific additional immunodeterminant present on the extracellular type 12 M-protein molecule.

Analysis for antiphagocytic component of extracellular M protein. To determine whether extracellular M proteins contained both the antiphagocytic determinants and the type-specific determinants found in other preparations of M proteins, M-protein preparations were analyzed by the indirect bactericidal assay. As Table 2 shows, both the L-form M protein and the protoplast M protein had the ability to completely remove type 12 opsonic antibody from rabbit immune serum. Therefore, these data, along with the immunodiffusion results, demonstrate that both the antiphagocytic and the type-specific components of M protein are present on extracellular M-protein molecules.

DISCUSSION

Our data demonstrate that in the presence of protease inhibitors, forms of group A streptococci lacking cell walls (i.e., protoplasts and L forms) secrete M-protein molecules that are both homogeneous in size and larger than previously reported for other M proteins. By using sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis, the molecular weights of the L-form and protoplast M proteins were determined to be 58,000, compared with 32,000 for detergent-extracted M protein. The question

TABLE 1. *Summary of gel diffusion data*

Antigen	Antiserum	Reaction[a]	Comments
L-form M protein	Type 12 specific	+	
Protoplast M protein	Type 12 specific	+	Line of identity with L-form M protein[b]
HCl-extracted M protein	Type 12 specific	+	Partial identity with L-form M protein[b]
Detergent-extracted M protein	Type 12 specific	+	Partial identity with L-form M protein[b]
L-form or protoplast M protein	Group A carbohydrate	−	
L-form or protoplast M protein	Group A variant carbohydrate	−	
L-form or protoplast M protein	Non-type 12 M proteins[c]	−	
L-form M protein	L-form M protein	+	
Protoplast M protein	L-form M protein	+	Line of identity with L-form M protein
HCl-extracted M protein	L-form M protein	+	Partial identity with L-form M protein
Detergent-extracted M protein	L-form M protein	+	Partial identity with L-form M protein
Group A carbohydrate	L-form M protein	−	
Group A variant carbohydrate	L-form M protein	−	

[a] +, Positive reaction; −, no reaction.

[b] See Fig. 1A.

[c] Types 1 through 6, 8, 9, 11, 13 through 15, 17 through 19, 22 through 34, 36 through 43, 46 through 63, and 65 through 67 (total of 57 non-type 12 antisera [unadsorbed]).

TABLE 2. *Indirect bactericidal test: absorption of type 12 M-protein opsonic antibodies with extracellular M proteins*

Serum	No. of colonies with the following inoculum:	
	42	20
Normal rabbit serum	Laked[a]	Laked
Stationary control	Laked	Laked
Rabbit opsonic serum[b]	1	0
Rabbit opsonic serum adsorbed with L-form M protein	Laked	Laked
Rabbit opsonic serum adsorbed with protoplast M protein	Laked	Laked

[a] Laked signifies confluent growth of organisms (>2,000 colonies).
[b] Antiserum 1.

now arises as to the size of the native M protein as it is found on the cell walls of group A streptococci. Two main possibilities exist. The first is that the molecular weight of M-protein molecules found on cell walls is in fact 58,000, and the second is that cleavage of the secreted 58,000-dalton molecules occurs, resulting in the insertion of 32,000-dalton molecules into the walls. If the first hypothesis is correct, then nonionic detergent extraction (a method which does not disrupt covalent linkages) may activate a wall or membrane-bound enzyme, resulting in cleavage of the native 58,000-dalton molecules and the release of 32,000-dalton fragments. In support of this hypothesis, two points concerning the detergent extraction procedure should be mentioned. First, unlike true detergent extractions, where one or two repetitions are necessary for the complete removal of a molecule or molecules, detergent extraction of M protein requires multiple repetitions in order to attain appreciable yields (6). This observation may be more in keeping with the concept of cleavage of the native M-protein molecule by a wall or membrane-bound enzyme. Second, only live cells (data not shown) or isolated cell walls from live cells (which are invariably contaminated with cytoplasmic membrane components [23]) may be used as starting materials for the detergent extraction method. M protein is not extracted easily with detergent from heat-killed streptococci, suggesting that its release may be the result of a heat-labile enzyme.

Furthermore, recent evidence from our laboratory indicates that type 6 M protein released from streptococcal cells by phage-associated lysin is a molecule of 50,000 daltons (Fischetti, unpublished data). This result supports the current data concerning the molecular weight of the secreted M-protein molecule (i.e., 58,000). The 8,000-dalton difference observed between these two molecules may be due to variations between M-protein types (type 6 versus type 12) or the presence of a leader sequence (i.e., signal segment) that is attached to the secretory M-protein molecule and is necessary for its passage through the membrane (4). However, a leader sequence would only account for 20 to 30 amino acid residues, and such residues are usually cleaved from a molecule after it transverses the membrane. Future sequence data will be required to determine whether the secreted M-protein molecule does in fact contain a signal sequence.

There are no readily available data in support of the second hypothesis (namely, that the 58,000-dalton molecule is cleaved, allowing a 32,000-dalton molecule to be inserted into the wall). Furthermore, a soluble 26,000-dalton M-protein fragment has not been reported in the growth supernatant of group A streptococci. However, as mentioned above, the molecular weight of the phage-associated lysin-extracted M protein is larger than the molecular weight of the detergent-extracted M protein, suggesting that a major cleavage of the secreted M protein does not occur before incorporation into the cell wall.

The intact secretory molecule demonstrated in these studies appears to conform to previous theories of M-protein structure. Using immunochemical data, Fox and Wittner (8) and Fischetti et al. (6) have suggested that M protein may have a subunit structure. More recent sequence analyses of purified M protein (2, 17) have clearly demonstrated that indeed there are repeating sequences within the M-protein molecule. The immunological data presented here demonstrated that even though there was an additional immunodeterminant(s) in the secreted M-protein molecule, only one-half of the rabbit antisera tested contained antibodies against this determinant(s). We can conclude cautiously that, in addition to the extra determinant(s), either the larger M protein contains repeats of the sequences present in the smaller molecule or portions of the intact protein are not immunogenic.

The observation that intact M protein did not react with any other unabsorbed M-protein type-specific antiserum suggests the absence of a common determinant among serotypes in the larger molecule. However, since the heterologous antisera used in this study were prepared against whole heat-killed organisms and not purified native M protein, a common determinant(s) may be present but in a position on the molecule that is buried within the cell wall matrix. Thus, because it has been demonstrated that group A streptococcal cell walls are highly

resistant to degradation in vivo by the phagocytic system (22), this determinant may be masked during antibody production. Future immunochemical studies with secretory M-protein molecules from various types should clarify this point.

In summary, we isolated intact M-protein molecules from the extracellular fluids of organisms lacking cell walls (i.e., L forms and protoplasts), and these molecules had an apparent molecular weight of 58,000. Also, these molecules had the same immunological capacities previously described for M proteins extracted by other methods, except for the possibility of an additional immunodeterminant(s). The demonstration of the existence of an intact M-protein molecule should permit experiments designed to investigate the organization of M-protein molecules in streptococcal cell walls. Either intact M-protein molecules are bound covalently to the cell walls by linkage to a heretofore unknown portion of the M-protein molecule, or M proteins are incorporated into walls through a noncovalent insertion process. The nature of this association is currently under investigation.

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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. Seyer, and A. H. Kang.** 1978. Repeating covalent structure of streptococcal M protein. Proc. Natl. Acad. Sci. U.S.A. **75**:3163–3167.
3. **Bleiweis, A. S., W. W. Karakawa, and R. M. Krause.** 1964. Improved technique for the preparation of streptococcal cell walls. J. Bacteriol. **88**:1198–1200.
4. **Blobel, G., P. Walter, C. N. Chang, B. M. Goldman, A. H. Erickson, and V. R. Lingappa.** 1979. Translocation of proteins across membranes: the signal hypothesis and beyond. Symp. Soc. Exp. Biol. **33**:9–36.
5. **Cohen, J. O., H. Gross, and W. K. Harrell.** 1977. Immunogenicity and characteristics of M protein released by phage-associated lysin from group-A streptococci types 1 and 23. J. Med. Microbiol. **10**:179–194.
6. **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.
7. **Fischetti, V. A., J. B. Zabriskie, and E. C. Gotschlich.** 1974. Physical, chemical, and biological properties of type 6 M protein extracted with purified streptococcal phage-associated lysin, p. 26–36. *In* M. J. Haverkorn (ed.), Streptococcal diseases and the community. Excerpta Medica, Amsterdam.
8. **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.
9. **Freimer, E. H., R. M. Krause, and M. McCarty.** 1959. Studies of L forms and protoplasts of group A streptococci. I. Isolation, growth, and bacteriologic characteristics. J. Exp. Med. **110**:853–874.
10. **Hryniewicz, W.** 1976. Streptococcal L forms and some of their properties. INSERM **65**:39–56.
11. **Kantor, F. S., and R. M. Cole.** 1960. Preparation and antigenicity of M protein released from group A, type 1, streptococcal cell walls by phage-associated lysin. J. Exp. Med. **112**:77–96.
12. **Karakawa, W. W., J. Rotta, and R. M. Krause.** 1965. Detection of M protein in colonies of streptococcal L forms by immunofluorescence. Proc. Soc. Exp. Biol. Med. **118**:198–210.
13. **Krause, R. M.** 1958. Studies on the bacteriophages of hemolytic streptococci. II. Antigens released from the streptococcal cell wall by a phage-associated lysin. J. Exp. Med. **108**:803–821.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
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.** 1959 Persistence of type-specific antibodies in man following infection with group A streptococci. J. Exp. Med. **110**:271–292.
17. **Manjula, B. N., and V. A. Fischetti.** 1980. Tropomyosin-like seven residue periodicity in three immunologically distinct streptococcal M proteins and its implications for the antiphagocytic property of the molecule. J. Exp. Med. **151**:695–708.
18. **March, S. C., I. Parikh, and P. Cuatrecasas.** 1974. A simplified method for cyanogen bromide activation of agarose for affinity chromatography. Anal. Biochem. **60**:149–152.
19. **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**:1298–1315.
20. **Russell, H., and R. R. Facklam.** 1975. Guanidine extraction of streptococcal M protein. Infect. Immun. **12**: 679–686.
21. **Sharp, J. T., W. Hijmans, and L. Dienes.** 1957. Examination of the L forms of group A streptococci for the group-specific polysaccharide and M protein. J. Exp. Med. **105**:153–159.
22. **Smialowicz, R. J., and J. H. Schwab.** 1977. Processing of streptococcal cell walls by rat macrophages and human monocytes in vitro. Infect. Immun. **17**:591–598.
23. **van de Rijn, I.** 1980. Structure and immunochemistry of the group A streptococcal membrane, p. 161–176. *In* J. Zabriskie (ed.), Streptococcal diseases and the immune response. Academic Press, Inc., New York.
24. **van de Rijn, I., and R. E. Kessler.** 1980. Chemical analysis of changes in membrane composition during growth of *Streptococcus pyrogenes*. Infect. Immun. **26**: 883–891.
25. **van de Rijn, 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**:579–599.