

STREPTOCOCCAL M PROTEIN
EXTRACTED BY NONIONIC DETERGENT
II. Analysis of the Antibody Response to the Multiple
Antigenic Determinants of the M-Protein Molecule*

By VINCENT A. FISCHETTI‡

(From The Rockefeller University, New York 10021)

Streptococcal M proteins are antiphagocytic molecules varying immunologically from type to type while maintaining an identical biological effect. The virulence of the group A streptococcus is directly related to the presence of the M antigens on the cell surface and resistance to infection by these organisms is dependent on the presence of opsonic antibodies directed towards the M molecules. Various methods such as latex agglutination (1), hemagglutination (2, 3), complement fixation (4), mouse protection (5), capillary precipitation (6), long-chaining tests (7), and radioimmunoassays (8, 9), have been used to determine the immune response to the M antigens. Despite this variety of alternative methods, the only generally acceptable and reliable measurement of type-specific opsonic antibodies in the serum is the standard bactericidal test (5). The occurrence of cross-reactions has precluded the replacement of the cumbersome bactericidal assay for the measurement of type-specific immunity to streptococcal infection with one of the other *in vitro* techniques.

The reason for these difficulties may be twofold: (a) the state of the M antigen used in the assay, and (b) the inherent nature of the protein antigen itself. The occurrence of cross-reactions has usually been attributed to unrelated antigens physically or chemically associated with the M protein (10-12). Although the M preparations used in many of these studies may well have contained other cellular antigens, an alternate explanation for these cross-reactions may be based on the presence of common regions in the polypeptide chain of certain M types (13-16).

In the present communication, a radioimmunoassay and solid-phase radio-competitive inhibition experiments are used to examine the antibodies developed against M6 protein in both human beings and hyperimmunized rabbits. By these techniques, the nature of a type-specific opsonic response and its relation to cross-reactions were examined on a molecular level.

Materials and Methods

M Protein. Purified antiphagocytic M6-protein molecules were prepared as previously described (17) from detergent-extracted streptococcal cell walls.

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Indirect Bactericidal Test. Bactericidal test procedures and analysis of results were as described previously (17). Results were scored as follows: <100 colonies = 4+ opsonic antibodies, 101-500 colonies = 3+, 501-1,000 colonies = 2+, > 1,000-partially laked = 1+, laked plate = 0.

Antisera. Type-specific antisera were prepared in rabbits by inoculation of whole, heat-killed streptococcal vaccines (18). Unabsorbed and absorbed antisera were prepared as described by Rotta et al. (18).

Human type 6 antiserum was from an individual who had high titer opsonic antibodies to type 6 streptococci. This individual had no history of rheumatic fever or nephritis.

Antisera against purified M protein was prepared by inoculation of 100 μ g of M6 protein (antiphagocytic molecular form [17]) in complete Freund's adjuvant at multiple sites in New Zealand red rabbits. Rabbits were boosted intravenously 1 and 2 mo after the initial immunization with 100 μ g of alum-precipitated M protein. Rabbits were bled 2 wk after each booster immunization and at monthly intervals thereafter.

Radioimmunoassay (RIA): Purified streptococcal M protein (20 μ g/100 μ l) was labeled with 125 I by the chloramine-T method of Greenwood et al. (19) with modifications by Gotschlich et al. (20). Labeled protein was separated from free 125 I by passing the solution over a 0.7 x 30-cm column of Sephadex G-10 equilibrated with phosphate-buffered saline at pH 7.4. 125 I-Labeled M protein was adjusted to a concentration of 300 ng/ml in 1% bovine serum albumin (BSA) and used in a Farr antigen-binding assay employing 40% saturation of ammonium sulfate (saturated at room temperature) to precipitate the immune complex (21). The double-label technique of Gotschlich (22) was employed. All sera were heat-inactivated for 30 min at 56°C and, when necessary, serum dilutions were performed in heat-inactivated fetal calf serum (FCS). Volumetric measurements were done with either Eppendorf pipettes (Brinkmann Instruments, Inc., Westbury, N. Y.) or with the use of an automatic pipette system (Micromedic Systems, Inc., Horsham, Pa.). All tests were done in duplicate and binding results varying >5% between duplicates were repeated.

Briefly, to a microtiter plate (Linbro IS MVC 96, Linbro Chemical Co., New Haven, Conn.) was added 10 μ l of test serum or a dilution thereof, 20 μ l of 1% bovine serum albumin, and 10 μ l of 125 I-labeled M antigen containing 22 Na. After equilibration overnight at 4°C, 40 μ l of 80% saturated ammonium sulfate (saturated at room temperature) was added and the plate placed at 4°C for 1 h. At this time, the precipitate was centrifuged for 20 min at 1,600 g and the majority of the supernate removed and counted in a Packard two-channel gamma spectrometer (model 3022, Packard Instrument Co., Inc., Downers Grove, Ill.) with one channel set to count 22 Na and the other, 125 I. The iodine count represents the antigen remaining unbound in the supernate in relation to the 22 Na counts. With these data, the exact amount of antigen bound to antibody can be calculated (22).²

Solid-Phase Radiocompetitive Inhibition. To determine if different immune sera would compete for the same or different sites on the M antigen, human serum or rabbit immune serum was bound to *N*-hydroxy-succinimide-activated Sepharose³ (23). The beads were diluted such that 20 μ l would bind between 90 and 100% of the radiolabeled M antigen at a concentration of 100 ng/ml. To a microtiter plate 20 μ l of serum or a dilution thereof, which was to act as inhibitor, was mixed with 20 μ l of 125 I-labeled M protein in 1% BSA containing 22 Na as a volume marker and the mixture allowed to stand at room temperature for 1 h. To this was then added 20 μ l of Sepharose beads bound with the appropriate serum. The plates were sealed and agitated on a Boerner shaker at 4°C for 48-72 h. At this time, the beads were sedimented at 300 g for 10 min and the majority of the supernate removed and counted. The bindings were calculated as described above (RIA). Percent inhibition was calculated from the binding observed in control tubes containing FCS or normal rabbit serum as inhibitor. Results were expressed as percent inhibition, calculated from the formula: $100 \times (\text{total antigen bound} - \text{total antigen bound in presence of inhibitor}) / \text{total antigen bound}$. As with the RIA, all tests were performed in duplicate and results varying > 5% between duplicates were repeated. All inhibitions were repeated at least twice on separate occasions to assure consistency of results.

¹ Abbreviations used in this paper: BSA, bovine serum albumin; FCS, fetal calf serum; RIA, radioimmunoassay.

² Gruss, A., and E. C. Gotschlich. Manuscript in preparation.

³ Also referred to as solidified antiserum in this paper.

Antigen-Antibody Dissociation Analysis. The stability of the ^{125}I -M-anti-M complex was determined from the rate of dissociation of the complex by a modification of the procedure described by Grey (8). To wells in a microtiter plate was added 10 μl of radiolabeled M6 protein at 300 ng/ml, containing ^{22}Na as a volume marker (22), 20 μl 1% BSA and 10 μl of antiserum. The antiserum concentration was such that 10 μl would bind $\cong 80\%$ of the antigen in the system. For each serum tested, five sets of duplicates were prepared which were allowed to equilibrate overnight at 4°C. At this time, 10 μl of unlabeled M6 protein was added to each well at a 100-fold excess of the radiolabeled M6 protein. At timed intervals (0, 1, 2, 4, 8, 24 h) after the addition of the excess M protein, 50 μl of 80% saturated ammonium sulfate (saturated at room temperature) was added to each set. The plates were then mixed, allowed to stand at 4°C for 1 h, and then centrifuged at 1,600 g to sediment the precipitate.

An aliquot of the supernate was removed, counted, and the percent of ^{125}I -M6 protein bound determined as described above (RIA). Percent dissociation was calculated by the formula:

$$\% \text{ dissociation} = \frac{\% \text{ppt}^4 (\text{time } \times) - \% \text{ppt} (\text{FCS}^5)}{\% \text{ppt} (\text{time } 0) - \% \text{ppt} (\text{FCS})} \times 100.$$

Absorption of Type-Specific Precipitating Antibodies. Type 6 opsonic rabbit serum was absorbed to remove only the type-specific precipitating antibodies as previously described (17). 120 μg of type-specific M-protein molecules bound to activated Sepharose (23) was added to 1 ml of hyperimmune M6 opsonic rabbit serum and the mixture rotated end over end at 4°C for 18 h. The beads were then sedimented at 1,500 g for 10 min and 0.5 ml of the absorbed serum was removed and reabsorbed with 60 μg of M protein under the same conditions as the first absorption. Serum absorbed in this way exhibited no precipitating reactivity by Ouchterlony or capillary precipitation with M6 protein. The absorbed serum however, maintained its opsonic activity in the indirect bactericidal test.

Results

Human Antibody Response to M Protein Measured by RIA. In contrast to acid-extracted M protein which precipitates with as little as 33% saturation of ammonium sulfate (16, 24, 25), detergent-extracted M protein will not begin to precipitate until a saturation of 60% has been attained. Taking advantage of this characteristic, purified antiphagocytic⁶ M6-protein molecules (17) were radiolabeled and utilized in an RIA using 40% saturation of ammonium sulfate to precipitate the immune complex (21). It was found that over a limited range (25–80% binding), a linear relationship exists between the percent of radiolabeled antigen bound and the logarithm of the antibody concentration for both human serum containing type 6 antibodies and rabbit type 6 immune serum.

Using this immunological tool, we attempted to determine whether binding could be correlated with the presence of opsonic antibodies in human serum. 24 human sera⁷ were separated into two groups: 10 sera containing no opsonic antibodies against type 6 streptococci, and 14 sera with opsonic antibodies, as measured by the indirect bactericidal assay employing normal human blood. When these sera were analyzed in the antigen-binding assay for their ability to bind radiolabeled M6 protein at 300 ng/ml (Fig. 1), all except two of those sera which had no opsonic antibodies bound <25% of the radiolabeled M6 antigen. Those sera containing type 6 opsonic antibodies bound between 40 and 80% of the

⁴ % ppt stands for percent counts in precipitate.

⁵ FCS for human system and normal rabbit serum for rabbit system.

⁶ Antiphagocytic molecules represent those M-protein molecules able to absorb opsonic antibodies from type-specific opsonic sera (17).

⁷ Some of these sera which were from individuals involved in a type-6 outbreak, were kindly supplied by Dr. Jean P. Widdowson, Cross-Infection Reference Laboratory, London, England.

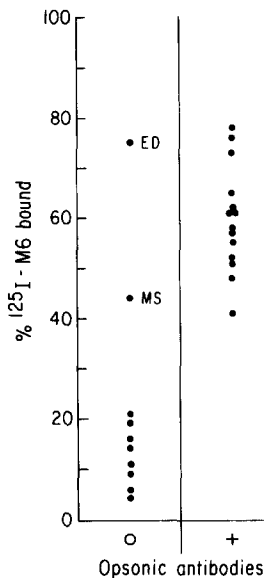


FIG. 1. Comparison between M6 opsonic and nonopsonic serum and their ability to bind ^{125}I -M6 protein in an RIA. 0 signifies no detectable opsonic antibodies to M6 streptococci in the indirect bactericidal test. Sera E. D. and M. S. were also tested in the direct bactericidal assay. + Signifies the presence of opsonic antibodies measured in the indirect bactericidal test.

radiolabeled M6 protein. Therefore, except for the two sera which had no opsonic antibodies⁸ yet bound 44 and 75% of the radiolabeled M6 antigen, a direct correlation could be seen between binding and the presence of opsonic antibodies to M6 streptococci.

The Relationship between Binding and Opsonic Antibodies. Further studies were designed to determine the nature of the binding of those sera which had no M6 opsonic antibodies yet bound the type 6 M-protein molecule (Fig. 1). It was postulated that these two sera might contain antibodies against the type-specific portion or cross-reactive sites of the M protein, but not towards the antiphagocytic moiety of this M antigen (17). To test this hypothesis, high-titered human M6 opsonic serum was bound to *N*-hydroxy-succinimide-activated Sepharose beads (23), and its ability to bind the radiolabeled antiphagocytic M6 protein was inhibited with varying concentrations of soluble opsonic sera as well as those sera which exhibited binding but no opsonic activity. As can be seen in Fig. 2, when binding of the M6 protein to the antibodies on the Sepharose beads was tested in the presence of various M6 human opsonic sera, 79–95% inhibition was observed at the highest serum concentration of all sera tested. These results indicate that nearly the complete complement of antibodies directed against the M6 molecule present in the solidified opsonic serum was also present in the soluble heterologous human opsonic sera. However, when the nonopsonic binding sera were used to inhibit the reaction, (Fig. 2, broken lines), only 25 and 9% inhibition was observed at the highest serum concentration,

⁸ These sera were tested in both the direct and indirect bactericidal tests.

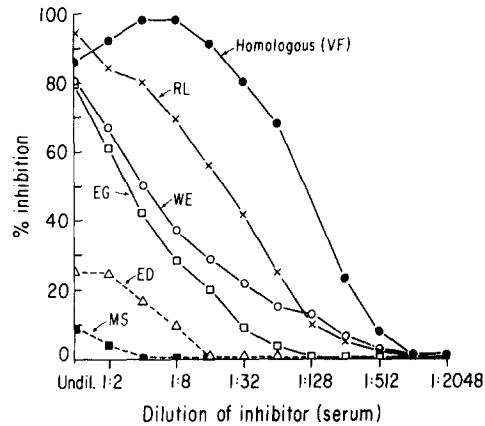


FIG. 2. Competitive inhibition of binding of ^{125}I -M6 protein to human opsonic serum (V. F.) on Sepharose beads. Serial dilutions of human M6 opsonic sera (—) and nonopsonic binding sera (----) were used as inhibitors.

suggesting that these sera lacked the complete complement of antibodies present in the opsonic sera.

The reciprocal experiment was also performed, in which one of the nonopsonic binding sera (E. D.) was immobilized on Sepharose beads, and its ability to bind the M6 protein inhibited with human opsonic serum as well as the homologous nonopsonic binding serum (Fig. 3). As can be seen, both the heterologous opsonic serum, along with the homologous nonopsonic serum, completely blocked the binding of the M6 protein to the nonopsonic serum on the beads. These results taken with the previous experiments suggest that the opsonic serum contains a population of antibodies not present in the nonopsonic but binding serum. The data are also consistent with the view that the antiphagocytic M-protein molecule contains multiple antigenic determinants (17), and only those antibodies directed against certain sites on the molecule are able to neutralize the antiphagocytic property of the antigen. To strengthen this hypothesis, similar experiments were performed with hyperimmune rabbit sera.

A battery of unabsorbed hyperimmune rabbit antisera directed against the heterologous streptococcal M types were analyzed for their ability to bind radiolabeled M6 protein in the RIA. It was found that 43% of these heterologous sera at a 1:11 dilution were able to bind the M6 antigen (Fig. 4), while 20% continued to bind this antigen at a 1:44 dilution. The homologous type 6 antiserum, however, bound >95% of the antigen at all these dilutions. When the cross-reactive sera were tested for their ability to precipitate the M6 protein or opsonize type 6 streptococci in a bactericidal test, they were found to be unreactive. Results such as these indicate that the cross-reactive sera contain antibodies directed against portions of the M6 molecule having regions in common with the cross-reactive M type, which does not encompass the antiphagocytic moiety. To determine the percent of the M6 molecule responsible for these cross-reactions with relation to the homologous M6 response, competitive inhibition experiments were performed. Various M6 rabbit antisera, as well as those heterologous sera which exhibited cross-reactions with the M6 molecule at 1:44

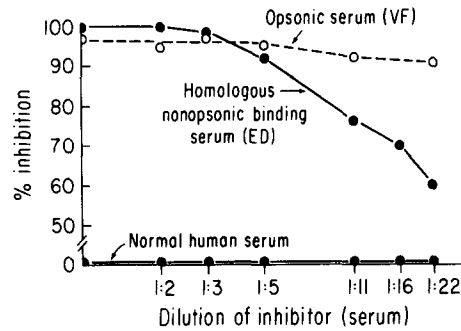


FIG. 3. Competitive inhibition of binding of ¹²⁵I-M6 protein to nonopsonic binding serum (E. D.) on Sepharose beads. Serial dilutions of the homologous nonopsonic binding serum (E. D.), heterologous opsonic serum (V. F.), and normal human serum were used as inhibitors.

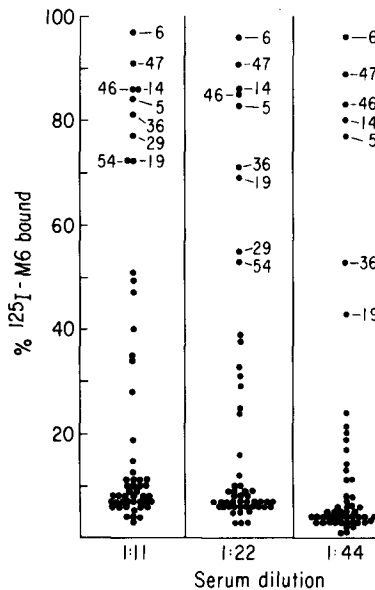


FIG. 4. Binding ¹²⁵I-M6 protein in an RIA with 1:11, 1:22, and 1:44 dilutions of unabsorbed heterologous rabbit sera. Numbers signify M types of those sera binding a significant amount of the M6 protein.

dilution (Fig. 4), and several sera exhibiting little or no cross-reactions even at a 1:11 dilution were used to compete with the solidified M6 antiserum for the antigenic sites on the radiolabeled M6 protein.

In the first experiments, M6 opsonic rabbit serum (R1940) was immobilized on Sepharose and dilutions of various M6 opsonic sera were used to compete for the binding of the radiolabeled M6 protein to the immobilized antibodies. As can be seen in Fig. 5, when high-titered (R1940 and R1941) and lower-titered (R4573) opsonic antisera prepared in different rabbits against whole M6 streptococcal vaccines were used as competitors, >95% inhibition was observed at high serum concentrations. Similarly, when a lower-titered opsonic serum prepared against

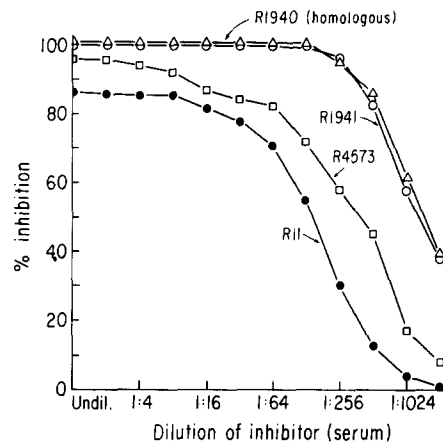


FIG. 5. Competitive inhibition of binding of ^{125}I -M6 protein to solidified M6 opsonic rabbit serum (R1940) with various M6 opsonic sera. Serial dilutions of high-titered M6 opsonic rabbit sera (R1940 and R1941) and low-titered serum (R4573) prepared with whole streptococcal vaccines were used as inhibitors. Dilutions of a lower-titered opsonic serum (R11) prepared by immunization with purified M6 protein was also used to inhibit binding of the M6 antigen to the antibodies on the Sepharose beads. Homologous signifies the serum which was bound to the beads and was also used as the soluble antiserum inhibitor.

purified M6 protein (R11) was used as a competitor, over 85% inhibition was observed up to a 1:8 dilution. On the other hand, as seen in Fig. 6, when the heterologous cross-reactive rabbit antisera were used to compete for the sites on the M6 protein, only partial inhibition was observed in all cases. Despite the fact that M47 antiserum had a high titer of cross-reactive antibodies (89%) against the M6 antigen in the binding assay (Fig. 4), these antibodies were directed to $\approx 40\%$ of the determinants recognized by the homologous M6 antibodies on the Sepharose beads. In contrast, M14 and M5 antisera, which bound $\approx 80\%$ of the M6 antigen in the RIA (Fig. 4), could compete for only $\approx 15\%$ of the sites on the M6 molecule. Similar results were also observed with M46 antiserum which blocked nearly 18% of the sites. Also evident from these data is the fact that several sera contain relatively high concentrations of cross-reactive antibodies, where multiple dilutions were required before a drop in titer was initiated. T36 antiserum, on the other hand, had high cross-reactive antibodies at high serum concentrations (Fig. 4) which could compete for the majority of the M6 sites. However, the level of these antibodies was low as demonstrated by the rapid drop in the dilution curve (Fig. 6). As expected, those sera which had little or no binding of the M antigen in the RIA (M38 and M42, as random representatives) also exhibited no inhibitory effect.

These results corroborate the data obtained with the human sera, indicating that opsonic serum contains a spectrum of multispecific antibodies directed against multiple antigenic sites along the M-protein molecule. In this spectrum, however, antibodies directed towards certain antigenic determinants seem to be necessary for opsonization. Cross-reactive antibodies, on the other hand, bind only to limited determinants on the M-protein molecule which do not seem to affect the antiphagocytic characteristic of the protein.

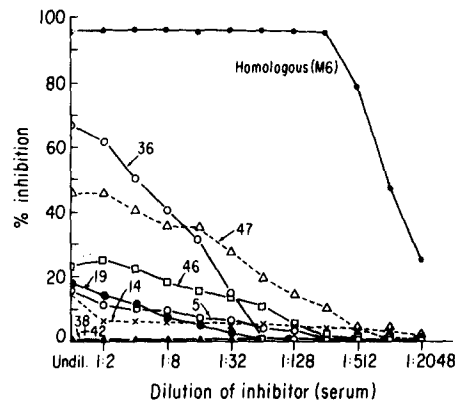


FIG. 6. Competitive inhibition of binding of ^{125}I -M6 protein to solidified M6 opsonic rabbit serum with heterologous cross-reactive rabbit sera. Serial dilutions of rabbit sera cross-reactive in the RIA (Fig. 4) were used as inhibitors. M6 antiserum was the positive control and noncross-reactive sera M38 and M42 were negative controls.

Opsonic Antibodies vs. Binding in the General Population: Since the above experiments on human sera were performed with sera from laboratory personnel and individuals with known M6 infections, the results may not reflect a true picture of the actual immune mechanisms involved; therefore, human sera from the general population were examined in a similar manner. Sera from 76 Trinidadian children, 5–12 yr of age, were tested for the presence of M6 antibodies by the RIA, screened for the presence of opsonic antibodies, and tested for the ability to compete against M6 opsonic serum for the antigenic sites on the M6 antigen. Fig. 7 illustrates that significant binding was observed with many of these sera when analyzed undiluted in the binding assay. However, the ability of these antibodies to bind the M6 antigen was reduced nearly to nonspecific background levels by a 1:11 dilution of the sera. When tested for the presence of opsonic antibodies, all the sera were found to be negative. Moreover, in the competitive inhibition assay, those sera exhibiting significant binding (>30%, undiluted) inhibited <28% of the antigenic sites on the M6 molecule with a mean inhibition of 10.2% (range 2.8–27.2%). These results indicate that even though many of the children tested had binding antibodies to the M6 antigen, the antibodies were of low titer or avidity, and reactive with only few sites on the M6 antigen.

A different picture was observed, however, when American adults between the ages of 21 and 28 were analyzed by these methods. As can be seen in Fig. 8, several sera contained binding antibodies to the M6 antigen which were not reduced by a 1:11 dilution. When the sera were tested for M6 opsonic antibodies, a lack of correlation was observed when compared with the bindings of undiluted sera, whereas good correlation was observed when compared with the sera diluted 1:11. The results indicate that except for sera 8976, 1331, 8935, 1413, and 8958, which exhibited significant binding but contained no M6 opsonic antibodies, those sera which bound >20% of the M6 antigen at 1:11 dilution contained M6 opsonic antibodies. Results of the competitive inhibition experiments on the high- and low-binding sera are also compiled in Fig. 8.

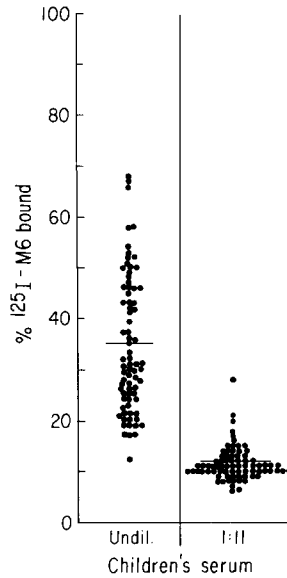


FIG. 7. Binding of ^{125}I -M6 protein in an RIA with sera from children in Trinidad. 76 sera were tested both undiluted and at a 1:11 dilution. Horizontal line signifies the mean binding in both dilutions. Mean age was 7.2 with a range of 5-12 yr of age. FCS binding was 8.3%.

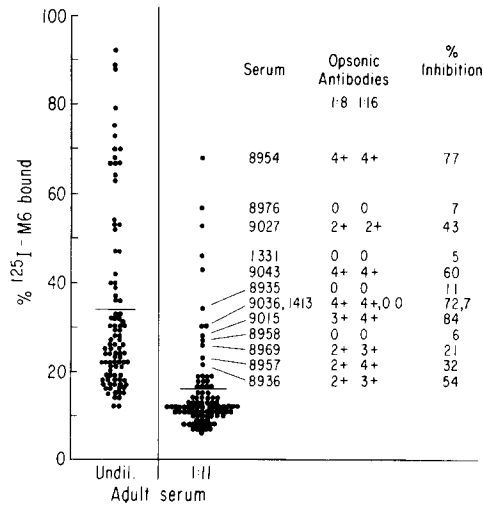


FIG. 8. Binding of ^{125}I -M6 protein in an RIA with sera from adults in the United States. Individual sera were tested both undiluted and at a 1:11 dilution. Indirect bactericidal results are tabulated individually beside those sera showing significant binding at a 1:11 dilution. The results are expressed as the amount of opsonic antibodies present in the serum based on the amount of growth seen after a 3-h rotation of two dilutions of M6 streptococci (1:8 and 1:16), (Materials and Methods). The results of the competitive inhibition assay performed on these sera are also indicated. No opsonic antibodies were observed in sera exhibiting <20% binding; inhibitions of <10% were also observed with these low-binding sera. Horizontal bar signifies the mean binding for both dilutions. The mean age of these individuals was 29.1 with a range of 21-38 yr of age. FCS binding was 8.3%.

It can be seen that those binding sera containing no opsonic antibodies could compete for only 11% or less of the antigenic determinants on the M6 protein. However, those sera with both binding and opsonic antibodies were able to block >20% of the sites on the M6 molecule with the majority of the sera inhibiting >50% of the determinants. Therefore, with the exception of sera 8969 and 8957 which inhibited 21 and 32%, respectively, the results are consistent with the results seen above; namely, that an opsonic response is demonstrated by antibodies directed against the majority of determinants on the M molecule.

The Role of Avidity in Opsonization. In an attempt to determine if the stability of the antigen-antibody complex (i.e. avidity) plays a role in promoting opsonization, the "off-rate" of the M-anti-M immune complex was measured. By the addition of a large excess of unlabeled M antigen to an equilibrated mixture of radiolabeled M6 protein and various sera, the dissociation rate of the complex can be analyzed.⁹ As can be seen in Fig. 9, when either rabbit (M6) or human opsonic sera (V. F. and 8954) were tested by this method, <31% of the ¹²⁵I-M6-anti-M6 complexes dissociated within 24 h. However, when cross-reactive sera were examined, striking differences were observed. The cross-reactive human serum W84 (from Trinidad) as well as the cross-reactive rabbit sera M19 and M47 exhibited >41% dissociation within the 1st h, indicating weak binding within the complex. In contrast, however, cross-reactive nonopsonic human (E. D.) and rabbit serum (M14) both exhibited antibody binding avidities equivalent to those seen in the opsonic sera. These results suggest that the avidity of an antibody for the M antigen is therefore not the sole factor necessary for opsonization. Other factors besides avidity, one of them being the site or sites bound by the antibody, also seem essential for opsonization to occur.

Binding Sites Necessary for Opsonization. In our previous publication (17) it was shown 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 its opsonic power. By using this selectively absorbed serum as an inhibitor in our competitive inhibition assay with the unabsorbed M6 opsonic serum immobilized on Sepharose, we were able to determine the nature of the specificity of the opsonic antibodies. The results presented in Fig. 10 indicate that antibodies directed towards 30% of the determinants on the M6 molecule could be removed by this absorption procedure, allowing for 70% inhibition to be observed. The data suggest therefore that the antibodies present in the absorbed opsonic serum were directed against up to 70% of the determinants on the M-protein molecule.

Discussion

Many of the difficulties encountered by Grey (8) and Anthony (9) in the use of M protein in an RIA have been overcome by detergent-extracted and purified M protein (17). ¹²⁵I-Labeled M6 protein at a concentration of 300 ng/ml was successfully used in a binding assay using 40% ammonium sulfate to precipitate the immune complex. In this system, <10% of the radiolabeled M antigen was

⁹ Certain qualifications of this statement will be alluded to in the discussion.

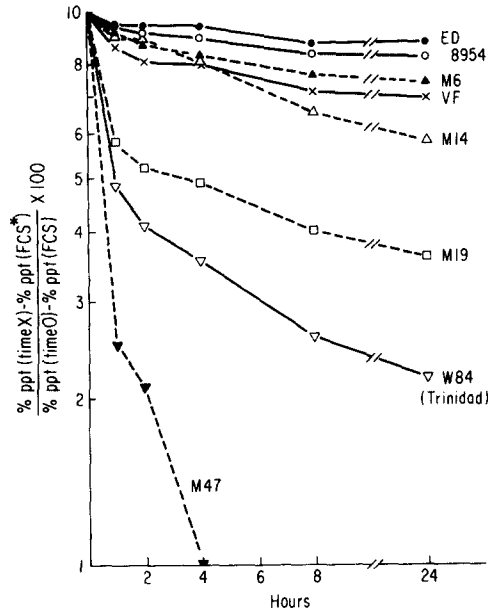


FIG. 9. Dissociation rates of ¹²⁵I-M6-anti-M6 complexes. Opsonic human sera (8954 and V. F.) as well as nonopsonic binding sera (E. D. and W84) were compared with rabbit opsonic (M6) and cross-reactive (M14, M19, and M47) antisera for their binding avidities for M6 protein.* FCS for human system and normal rabbit serum for rabbit system. % ppt, percent counts in precipitate.

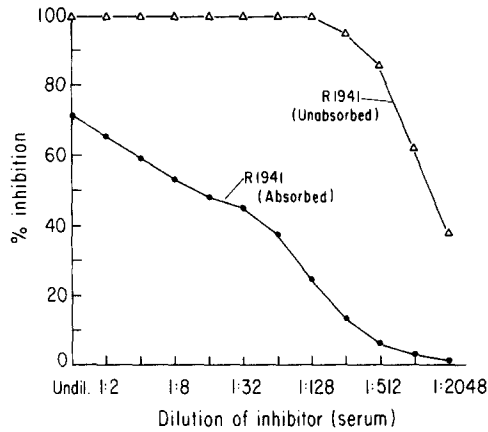


FIG. 10. Competitive inhibition of binding of ¹²⁵I-M6 protein by selectively absorbed serum. M6 opsonic rabbit serum (R1941) was absorbed with type-specific M-protein molecules to remove the type-specific precipitating antibodies without affecting the opsonic power of the serum (17). The unabsorbed serum was immobilized on Sepharose and dilutions of the homologous unabsorbed and absorbed sera were used to compete with the immobilized antibodies for the sites on radiolabeled M6 protein.

bound by FCS or normal rabbit serum, whereas >95% was bound by the homologous type-specific rabbit antisera.

Even though good correlation was observed between the binding capacity of human serum and its opsonic power in the M6 system, we have observed that

sera of certain individuals lacking opsonic antibodies do contain antibodies able to bind the M6 antigen. These results prompted us to consider the nature of a type-specific opsonic response to this multideterminant antigen with relation to cross-reactions.

Cross-reactions in hyperimmune rabbit sera have been described by several investigators (26, 27). Cross-absorption experiments between M12 and M3 by Fox and Wittner (15) indicated that these proteins contained areas in common which were responsible for cross-reactions in a hemagglutination assay. However, these antibodies were not opsonic for the heterologous type, suggesting that the antigenic portions of these M-protein molecules responsible for stimulating opsonic antibodies was not shared. Wittner (13), in a more recent publication, was able to separate passive mouse-protecting and hemagglutinating antibodies from opsonic antibodies by a specialized absorption procedure, again pointing to common antigenic determinants among certain M proteins.

By the use of the solid-phase radiocompetitive inhibition experiments described here, the mechanism of these cross-reactions may be observed on a molecular level. Before proceeding, however, the reader must be made aware that interpretation of these experiments was based on the following assumption: Since whole serum was used in the binding of the antibodies to the Sepharose beads, it was assumed that the proximity of one antibody to the next was such that an M-protein molecule could not be bound by two adjacent antibodies. If this is the case (and experimental results seem to bear this out), then the binding of a soluble antibody molecule to an antigenic determinant on the M protein will inhibit that protein from binding to the solid-phase antibody directed to that determinant.

The results presented here support the view that the antiphagocytic M molecule contains multiple antigenic determinants; i.e., some concerned with type-specificity and/or cross-reactivity, and some with antiphagocytosis (17). An antigenic structure such as this would give rise to a multispecific antibody population in the host. Assuming that the complete complement of antibodies to the M-protein molecule is present in the opsonic serum which is bound to the Sepharose beads, the small amount of inhibition brought about by the binding but nonopsonic sera (Fig. 2) indicates that these sera do not contain antibodies to all the determinants present on the M molecule. Since these antibodies can bind only to a limited number of sites on the M molecule (i.e. type-specific or cross-reactive), then other sites (i.e. antiphagocytic) would be free to bind to the opsonic antibodies on the Sepharose beads.

In contrast, when opsonic sera (Fig. 2) were used in the competitive inhibition assay, >79% of the antigenic determinants were blocked from binding to the solidified opsonic serum. These results indicate that a type-specific opsonic response to the M antigen is manifest by the production of antibodies directed against the majority of the antigenic determinants of that M antigen.

Binding and inhibition experiments performed with radiolabeled M6 protein on heterologous hyperimmune rabbit sera revealed similar results. The majority of these heterologous sera bound <20% of the M6 antigen in a standard RIA while certain sera contained antibodies which bound >20% of the M6 antigen even when diluted 1:44. In spite of the presence of high-binding cross-reactive

antibodies, these heterologous sera exhibited no opsonic or precipitating activity with the M6 system.

Results of the competitive inhibition experiments revealed that when various M6 sera were used to inhibit the binding of M6 protein to the solidified M6 opsonic rabbit serum, inhibitions of >85% were observed (Fig. 5). This amount of inhibition was consistent regardless of the type of M antigen used to raise the antibodies (i.e., whole streptococcal vaccine or purified M protein). In contrast, the heterologous cross-reactive sera were only able to compete for a limited number of determinants along the M6 molecule (Fig. 6) in spite of the high binding capacity of some of these sera (i.e., M47, M46, M14, M5, Fig. 4). Again, these results suggest that the antiphagocytic moiety of the M antigen is restricted to certain determinants along the molecule. Even though \approx 50 and 70% of the M6 determinants were bound by M47 and M36 antisera respectively, the sites responsible for the antiphagocytic effect were not affected by these two heterologous sera, as demonstrated by their inability to promote opsonization of M6 streptococci in a bactericidal test. The fact that these sera were of low avidity may help to explain these results (see below).

Turning now to the results obtained with sera gathered from the general population, it is well-known that children in rural areas of Trinidad are chronically infected by group A streptococci through impetiginous infections (28). Binding experiments to measure antibodies to type 6 streptococcal M protein revealed that even though M6 streptococcal infections are extremely rare in Trinidad, the majority of these sera when tested undiluted, had significant binding antibodies to this protein. However, a 1:11 dilution of the sera nearly reduced the binding to base-line levels (Fig. 7). On the other hand, none of the sera contained opsonic antibodies to M6 streptococci when tested in the bactericidal test. Moreover, when tested in the competitive inhibition experiments, even those sera which had high cross-reactive antibodies exhibited limited inhibition of the M6 antigen (<25%), suggesting that the M antigen of strains endemic to Trinidad may contain regions in common with the M6 antigen. The fact that the binding can be reduced by a 1:11 dilution of the serum suggests that these antibodies are of low titer and/or low avidity.

In contrast, several adult sera from individuals in the United States exhibited substantial binding to the M6 antigen when tested both undiluted and at a 1:11 dilution. When the sera were tested for opsonic antibodies to M6 streptococci, most of the sera which bound >20% of the antigen at a 1:11 dilution also contained opsonic antibodies. However, as in our previous results (see above), some of the sera which bound the M antigen did not contain opsonic antibodies. When the sera were tested by the competitive inhibition experiments, those with opsonic M6 antibodies inhibited >20% of the homologous reaction with the majority of these sera inhibiting >50% of the sites on the M antigen. On the other hand, the nonopsonic binding sera could only inhibit <12% of these determinants. As with the rabbit sera results, these data again suggest that an opsonic response to the M antigen is represented by the production of antibodies to the majority of the antigenic determinants on the M molecule.

The determination of the avidity of a particular serum by measuring the "off-rate" of an immune complex is a difficult problem when dealing with a multide-

terminant antigen. RIA methods used here in separating bound from free antigen (i.e. precipitation with ammonium sulfate) score an antigen as "bound" whether it has one or more antibody molecules bound to it and score it "free" only if no antibodies are bound. By this method, one can distinguish only two over-all states of the whole antigen; either "free" with no antibodies bound or "bound" with one or more of the antigenic determinants bound by antibody. Realizing this limitation, it was still quite evident that the avidity of the opsonic sera was substantially greater than those which were nonopsonic. It was also evident from these results that certain nonopsonic sera (E. D. and M14, Fig. 9) had at least one antibody whose avidity was equivalent to that found in the opsonic sera. This suggests that an antibody must possess special characteristics other than high avidity before it can be capable of opsonization. Grey (8) in 1962 observed similar dissociation rates on sera from rabbits immunized with group A streptococci. However, since no bactericidal tests were performed on these sera, direct comparisons with our results cannot be made.

Additional information concerning the sites bound by the opsonic antibodies was revealed by the competitive inhibition results using selectively absorbed sera. It was determined from these experiments that the type-specific precipitating antibodies present in rabbit serum were directed towards only 30% of the antigenic determinants on the M6 molecule whereas the remaining opsonic antibodies reacted with up to 70% of the sites. These results indicate that multiple antigenic determinants situated along the M-protein molecule must be bound by specific antibodies before opsonization occurs.

One can see by the results presented that a type-specific opsonic response to an M antigen is represented by the production of antibodies directed against the mosaic of antigenic determinants which comprise this antigen. The results also suggest that opsonization is not prompted by the mere binding of antibodies to the M antigen, but necessitates the binding of avid antibodies to a number of type-specific antiphagocytic determinants on the antigen. Furthermore, results of the competitive inhibition experiments on both rabbit and human opsonic sera indicate that an opsonic response is generally demonstrated by antibodies directed against the majority of the determinants on the M molecule. This suggests that the production of opsonic antibodies may be the culmination of the response to the antigen. Whether this is strictly a quantitative or qualitative effect is presently under investigation. Cross-reactive antibodies, on the other hand, are directed against a limited number of antigenic determinants and do not possess opsonic activity. If carefully and systematically utilized, these cross-reactions, in conjunction with structural data, may enable us to understand more clearly the relation between various M types and the nature of their antigenic shifts (29).

Summary

Purified streptococcal M protein extracted by nonionic detergent was used in an RIA and a solid-phase radiocompetitive inhibition assay to determine the nature of the immune response in both human beings and hyperimmunized rabbits to this complex antiphagocytic antigen. Results indicate that a type-specific response to an M antigen with the development of opsonic antibodies is

the result of antibodies directed against the majority of the antigenic determinants of the molecule. Cross-reactions between certain M types on the other hand, are represented by antibodies directed against only a small percentage of these antigenic determinants. Results also suggest that avidity may play a role in the action of opsonic antibodies. However, the data indicate that factors besides avidity (i.e. sites bound by the antibodies) also seem essential for opsonization.

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