Despite antigenic diversity, group A streptococcal M protein has been able to maintain its biological property of being antiphagocytic. The mechanism by which this type-specific antiphagocytic effect is exerted at the molecular level has yet to be elucidated. Whereas immunological cross-reactions do occur between certain M types (1-3), rarely do these cross-reactive antibodies afford cross-protection (2, 4, 5). Therefore, we decided to examine the relationship of serological cross-reactions to structural relatedness between three streptococcal M types (M6, M12, and M14). By comparing the peptide maps between cross-reactive and non-cross-reactive M types, it was discovered that cross-reactions are correlated with the presence of several common peptides within these M proteins, pointing to some degree of structural similarity between these types. On the other hand, the non-cross-reactive M types yielded only three common peptides, of which only one was common to the three proteins analyzed. In addition, the data suggest that molecular conformation may play an important role in both immunological and biological activities of the M antigens. Mapping results also indicated that a major product of tryptic digestion found in all three M proteins examined was free lysine, suggesting a common lys-lys and/or arg-lys sequence.

**Materials and Methods**

**Preparation of M Protein.** Types 6, 12, and 14 M proteins were prepared as previously described (6) using nonionic detergent as the extracting agent.

**Preparation of Tryptic Peptides.** 300 μg (≈ 10 nmol) of M protein were dialyzed against 0.1 M ammonium bicarbonate buffer, pH 8, and then lyophilized. The protein was resuspended in 100 μl of 0.2 M ammonium bicarbonate, pH 8.0, and 3 μg of TPCK-trypsin (Worthington Biochemical Corp., Freehold, N. J.) was added in a total of 3 μl. The solution was allowed to incubate at 37°C for 7 h, and it was then lyophilized, resuspended in 0.2 ml H2O, redissolved, and stored desiccated until use.

**Buffers.** Sodium citrate buffers were utilized to elute the peptides from the sulfonated polystyrene column. Sodium citrate buffers of 0.2 M, pH 3.1; 0.5 M, pH 4.0; 1.0 M, pH 5; and 1.5 M, pH 6, were prepared in fresh double-distilled water with the molarities based on the sodium ion concentration. The pH was adjusted with hydrochloric acid which was redistilled over sodium dichromate (7) to remove contaminating amines. All buffers contained 0.2% nonionic detergent.
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(octa-ethyleneglycol-dodecyl-ether) and 0.025% Merthiolate, and they were filtered through a 1.2-
μm pore size filter (Millipore Corp., Bedford, Mass.).

O-Pthalaldehyde reaction buffer was prepared by adding 100 mg of Floram (Durrum Instrument Corp., Sunnyvale, Calif.), dissolved in 1 ml 95% ethanol, to 1 liter 0.8 M borate buffer, pH 10.5, containing 2 ml 2-mercaptoethanol and 3 ml Brij-35.

To avoid contamination by amines from the air, all buffers were maintained under positive pressure with nitrogen which had been bubbled through concentrated H₂SO₄.

Analytical System and Operational Procedures. Sulphonated polystyrene ion exchange resin type PA35 (Beckman Instruments, Inc., Mountainside, N.J.) was packed under pressure into a 0.6 × 15-cm jacketed high-pressure column (Glenco Scientific, Inc., Houston, Tex.) with the temperature maintained at 50°C. The column was flushed with 5 ml 0.2 M NaOH and equilibrated with the 0.2 M citrate buffer, pH 3.1. The trypsinized protein sample, suspended in 0.5 ml of the pH 3.1 citrate buffer, was introduced into the column using an automatic sample loading device (Altex Scientific Inc., Berkeley, Calif.). The column was eluted with a linear gradient using four chambers of a nine-chambered Varigrad gradient maker (VirTis Co., Inc., Gardiner, N. Y.), with each chamber containing 75 g of one of the four citrate buffers in the order pH 3.1, pH 4.0, pH 5.0, and pH 6.0. The buffer gradient (=300 ml) was delivered to the column utilizing a high-pressure Milton Roy pump (Laboratory Data Control Div. Milton Roy Co., Riviera Beach, Fla.) at a flow rate of 24 ml/h (150-180 lb/in² pressure).

The column was monitored using a Technicon bubble-separation Autoanalyzer system similar to the one described by Catravas (8) with extensive modifications. 20% of the column effluent was combined with two volumes of the o-phthalaldehyde buffer, and the mixture was passed through a 3-min delay mixing coil. The reaction mixture was de-bubbled and passed through a 30-cm cooling jacket maintained at 15°C (to avoid the evolution of small bubbles in the 10-μl micro flowcell) and read by a Fluorimeter (American Instrument Co., Silver Spring, Md.). The flowcell chamber was also maintained at 15°C using a constant-temperature circulating cooling bath. To increase the stability and the sensitivity of the instrument, the bulb housing of the Fluorimeter was adapted to accept an Oriel low pressure UV lamp C-13-61 (Oriel Corp. of America, Stamford, Conn.). The flowcell was flanked by a Corning 7-60 primary filter and a Wratten 2A secondary filter (American Instrument Co., Silver Spring, Md.). The photomultiplier outputs were connected to a Linear 2-pen recorder (Linear Instruments Corp., Irvine, Calif.) and an Autolab system AA integrator (Spectra-Physics Inc., Mountain View, Calif.).

After each run (12 h), the column was flushed with 0.2 N NaOH for 15 min, and reequilibrated with the 0.2 M pH 3.1 starting buffer. By this method, more than 80 runs could be performed before the resin had to be removed and reconditioned as described in the Beckman Technical Bulletin for the PA35 resin.

Analysis of Data. Elution times of each peak were monitored by the Autolab AA integrator. Peaks having elution times differing by ±2.3 min (in a total run time of 12 h) were considered identical. This time is based on the standard deviation of peak elution times obtained upon repetitive protein and amino acid standards over a period of six mo.

Isolation and Analysis of Free Lysine Peak. The majority of the sample from the PA35 column (≈80%) was passed through a delay coil with a transit time identical to the analyzed portion of the stream. The sample was collected by a fraction collector connected by an event marker to the recorder allowing for fractions to be correlated with peptide peaks. Fractions corresponding to the lysine peak were desalted as described by Drèze et al. (9). After lyophilization, half the sample was acid-hydrolyzed before amino acid analysis, and half was analyzed directly on a Durrum D500 amino acid analyzer.

Control Proteins. Tryptic peptide maps of 10-50 nm of staphylococcal protein A (Pharmacia Fine Chemicals, Piscataway, N.J.) and hemoglobin S (kindly supplied by Dr. James Manning of The Rockefeller University) were prepared as described for the M protein.

Results

Comparison of Peptide Patterns between Immunologically Related and Unrelated M Types. Recent radioimmunoassay studies from this laboratory utilizing M6 protein as the radiolabeled antigen and unabsorbed rabbit antisera directed against heterologous M types revealed that several antisera exhibited
significant cross-reactions with the M6 antigen (10). The results suggested that these cross-reactive M types may have regions in common with the type 6 antigen, thus causing a certain degree of immunological cross-reaction with the M6 protein. In addition, competitive inhibition data indicated that unlike the M6 antisera which bound to the majority of the determinants on the M6 antigen, the cross-reactive antisera bound only to limited regions of the M6 antigen and were unable to neutralize the antiphagocytic effect of the M6 protein (10).

To determine the extent of structural similarity between cross-reactive and non-cross-reactive M types, as well as gaining insights as to the structural mechanisms involved in the common antiphagocytic effect of M protein, trypic peptide maps of the 28,000–35,000 dalton molecules of two selected M proteins were compared to that of the M6 protein. M14 protein was chosen as a highly cross-reactive antigen since M14 antiserum at a 1:11 dilution bound up to 86% of the M6 antigen in a radioimmunoassay (10). On the other hand, M12 protein was selected as an essentially non-cross-reactive antigen since M12 antiserum bound only 15% (background levels) of the M6 antigen under the same conditions.

Fig. 1 reveals that trypic peptide maps of the cross-reactive M6 and M14 proteins contain 9 of 16 peaks in common, suggesting a certain degree of structural relatedness between these two M types. Conversely, Fig. 2 illustrates that the M12 protein has only 4 peaks in common with the M6, indicating limited structural similarities between these two non-cross-reactive M proteins. In addi-
tion, M6, M12, and M14 all release free lysine upon tryptic digestion (peak 10), suggesting that each contains lys-lys, or arg-lys sequences, or both. Surprisingly, the data also reveal that peptide 14 is the only peptide common to all three M types examined.

With relation to the free lysine peak, it was found that when the M protein chromatograms were compared with those of amino acid standards, no other peptide peak corresponded with any of the other standard amino acids. To further confirm the release of free lysine from a lys-lys sequence, tryptic peptides of hemoglobin (which has a known lys-lys sequence in both alpha and beta chains [11]) run on the peptide analyzer also exhibited a free lysine peak. In addition, amino acid analysis of the material in the lysine peak of the M protein digest verified the presence of only lysine. The quantity of lysine released by trypsin, however, was difficult to assess since tryptic digestion is not quantitative. However, it may be safe to assume that at least one lys-lys or arg-lys sequence is present in these three M protein molecules.

When peptide maps from different lots of the same M type were compared, reproducible patterns were observed, even down to the shoulders and shape of the individual peaks. Also, when tryptic peptide maps of control proteins such as staphylococcal protein A and hemoglobin were compared to those of the three M proteins, no similarity in peptide patterns was observed. Occasionally, however, a control peptide peak would overlap in elution time with a peptide in an M protein digest, suggesting that both peptides were of similar charge and/or sequence.

In the M protein chromatograms, no peptides were observed beyond 8 h of a 12-h run, and few peptides emerged before 2 h. Tryptic maps of the hemoglobin control, on the other hand, revealed several major peptides in these regions, suggesting that the peptides released by trypsin from the M-protein molecule are not generally of a highly or weakly charged nature.

Discussion

It is now well established that immunochemical cross-reactions between related proteins usually correspond directly with structural similarity (12). On
the other hand, it has also been shown that the presence or absence of cross-reactions may be influenced by conformational changes within similar molecules (13); thus, cross-reactions between closely related proteins may not only be a consequence of sequence similarities. For instance, lysozyme and alpha lactalbumin, two proteins closely related in both primary structure (14) and three dimensional configuration (15, 16), exhibit no immunological cross-reactions (17). In spite of major primary sequence similarities between these two molecules, conformational configurations seem to be a major factor controlling the nonidentity of the antigenic determinants (17). Additionally, human, beef, lamb, goat, and sperm whale myoglobin molecules, having similar sequences and biological function, exhibit little or no immunological cross-reactions (13). Anomalies such as these could only be explained by inherent differences in conformation. For instance, it has been shown that antibodies raised against many types of native proteins seem to be directed against conformational structures rather than sequential determinants (18, 19).

In the case of the three M protein molecules examined in this report, it appears that the immunoochemical cross-reactivity observed (10) may be influenced to some degree by the structural relatedness of the proteins involved. Despite the extent of cross-reactivity and structural similarity between M6 and M14 proteins, solid-phase radiocompetitive inhibition assays (10) demonstrated that M14 antiserum could bind to only 5% of the M6 determinants recognized by the M6 antiserum. This suggests that the conformational relatedness between these two M molecules with respect to the antigenic determinants available may actually be less than what peptide map analyses seem to indicate.

In our attempt to understand the mechanism by which the M protein molecule retains its antiphagocytic function while diverging antigenically, we argued that any minor sequence changes within the protein could promote changes in the immunological appearance of the molecule. However, these shifts would not cause major conformational alterations of the antiphagocytic portion of the protein. Major sequence changes, on the other hand, could eventually result in the disruption of the antiphagocytic function of the M-protein molecule. Hence, when these studies were initiated it was anticipated that the structural differences between M-protein types would be minor, with the changes being concerned primarily with those areas of the protein involved with its immunological reactivity (i.e., "constant" regions concerned with antiphagocytic determinants, and "variable" regions controlling the type specificity). It can be seen from these mapping results that even though all three M protein molecules have antiphagocytic determinants (evidenced by their ability to remove type-specific opsonic antibodies from serum), M6 and M12 have little structural relatedness. In other words, although M6 and M12 appear quite different both immunologically and structurally, their conformational appearance to an approaching phagocyte may be similar. These results suggest that biological activity may be based on conformational relatedness rather than primary sequence similarity (i.e., a tertiary structure exposing the same functional groups despite sequence variation).

This type of mechanism may have evolved by the action of selective pressure on the M-protein molecule. Without an alternate host, the group A streptococcus is faced with the dilemma of changing its immunological appearance while main-
taining the ability to persist in man. Therefore, during evolutionary changes in the M antigen, possibly dictated by immunological pressure, those configurational alterations that accomplished both antigenic shifts without the loss of biological conformation could eventually survive.

The only consistency observed between all the maps in these studies is the release of free lysine by the action of trypsin and the presence of peptide 14. The former suggests the presence of either a lys-lys or arg-lys sequence, or both, within the protein. Since lysine is one of the major amino acids found generally in M protein by us (6) as well as others (20–22), this result is not unexpected. The presence of peptide 14 in all three M types, on the other hand, could suggest that this sequence may be involved in the antiphagocytic effect of the M molecules. However, no general opsonic antibody has ever been demonstrated, even against a limited number of M types, and this argues against a common antigenic sequence having the antiphagocytic property. In this regard, we have clearly shown that only when antibodies are directed against the majority of the determinants on the M protein will it neutralize the antiphagocytic effects of the molecule. On the other hand, avid antibodies binding to a limited number of determinants on the protein do not affect its biological property (10). Based on this data, it seems likely that through conformational shifts, the proximity of certain amino acids creates the required antiphagocytic environment for the M molecule, and that the spatial array of the determinants creating this environment may be such that several antibody molecules are necessary for its neutralization (10). An alternate hypothesis would be the presence of an antiphagocytic sequence which does not stimulate the production of antibodies by virtue of its similarity to a mammalian sequence. In this case, antibodies developed against the antigenic domains adjacent to this sequence would sterically neutralize the antiphagocytic effect of the active region. Sequence and immunochemical data are presently being accumulated in an attempt to answer these questions more conclusively.

Summary

Three immunologically cross-reactive and non-cross-reactive streptococcal M proteins were analyzed by a chromatographic tryptic peptide mapping system. The results indicate that cross-reactions correlate with the extent of structural similarity among the M protein molecules analyzed. The data also reveal that free lysine is released by the action of trypsin from these three M proteins, suggesting a common lys-lys or arg-lys sequence. In addition, only one peptide has been found to be common within all three M types. This limited structural relatedness among the three M proteins examined indicates that sequence variation plays a major role in the immunological specificity of the M antigens. However, despite sequence variation, all M protein molecules have a common antiphagocytic activity. The fact that no common opsonic antibody has yet been found, even against limited M types, argues against this biological activity being solely the result of a common sequence. Based on these data, it is suggested that the antiphagocytic effect of M protein may be due to a conformationally created environment on the surface of the molecule which is selected by both immunological and biological pressure.
The author wishes to thank Doctors E. C. Gotschlich, J. B. Zabriskie, Maclyn McCarty, I. van de Rijn, and J. W. Tauber, and Ms. E. De Falco for their suggestions in the preparation of this manuscript. I also wish to acknowledge Ms. Nancy Fertitta for her superb technical assistance in these experiments, and Ms. Sheenah McManus for her excellent assistance in the isolation and analysis of the amino acids.

Received for publication 28 December 1977.

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