AUTOIMMUNE SEQUENCE OF STREPTOCOCCAL M PROTEIN SHARED WITH THE INTERMEDIATE FILAMENT PROTEIN, VIMENTIN

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Acute proliferative glomerulonephritis (AGN) after streptococcal infections is thought to be an autoimmune disease. Although it is known that the disease is triggered by infections with so-called nephritogenic strains of Streptococcus pyogenes, the pathogenesis has been poorly understood mainly because the antigens involved have not been identified.

Recently it has become increasingly clear that the M protein molecules protruding from the surface of group A streptococci contain epitopes capable of eliciting autoimmune reactions with specific host tissues. Shared sequences of types 5, 6, and 19 M proteins have been shown to evoke antibodies that react with several myocardial and skeletal muscle proteins, including myosin, tropomyosin, and other as yet unidentified proteins (1-6). Most recently, Gorony-Bermes et al. (7) showed that an mAb raised against human renal cortex crossreacted with types 6 and 12 M proteins, providing evidence that M proteins of nephritogenic strains share antigenic determinants with renal glomeruli. These observed crossreactivities have hampered the development of a vaccine against streptococcal infections because of the fear that autoimmune epitopes in the M protein molecule may cause rather than prevent the poststreptococcal infectious sequelae, acute rheumatic fever, and acute glomerulonephritis.

The M protein molecules radiate as α-helical coiled-coil fibrils from the bacterial cell surface and this protein is the major virulence determinant of S. pyogenes bacteria (8, 9). The fibrils render the organisms resistant to recognition and ingestion by phagocytes of the nonimmune host (10). The resistance to phagocytosis is overcome only by antibodies directed towards protective epitopes on the M protein molecule (8). In our search for protective antibodies against type 1 streptococci, a serotype associated with the development of both acute rheumatic fever and acute proliferative glomerulonephritis (11), we identified protective as well as tissue crossreactive epitopes in the NH2-terminal part of type 1 M protein (12). We have shown that a tetrapeptide sequence of type 1 M protein specifies a renal glomerular crossreactive epitope. Antibodies against this region reacted with renal glomeruli in a
cellular distribution as demonstrated by immunofluorescence tests of frozen sections of human renal cortex (12).

Here we report that antibodies directed against a renal glomerular crossreactive epitope of the NH₂-terminal region of type 1 M protein react with mesangial cells cultured from isolated glomeruli. The intermediate filament protein vimentin, which shares limited amino acid sequences with type 1 streptococcal M protein, is identified as the autoimmune target within these cells.

Materials and Methods

Antigens and Antibodies. Purified bovine vimentin (57 kD), purified chicken desmin (53 kD), and a polyclonal antisemur to human vimentin were purchased from ICN Immunobiologicals (Lisle, IL). mAbs to human vimentin and human desmin were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Antiserum against the synthetic peptide SM1(1-19) (23-26)C of type 1 M protein were raised in a previous study (13) in which the peptide was named SM1(1-20) (24-26)C. We have since renamed the peptide SM1(1-19) (23-26)C, because of the M1 sequence, either deletion yields the same peptide sequence and the tetrapeptide represented by residues 23-26 specifies the renal glomerular crossreactive epitope (12). Antiserum 8652, which showed the strongest crossreactivity with human renal glomeruli, was affinity purified over a column of Affigel 10 (Bio-Rad Laboratories, Richmond, CA) to which the synthetic peptides SM1(1-19) (23-26)C or SM1(23-26)C had been covalently linked, as described by Kraus and Beachey (12).

Culture of Rat Glomerular Mesangial Cells. Glomeruli were isolated and cultured mesangial cells were obtained as described (14-16). Briefly, kidneys were removed from ~100-g female Wistar rats aseptically and the cortices were incised into small pieces and gently mashed by the finger tip. The suspension of mashed tissue was passed through stainless sieves at 60, 80, 120, and 280 meshes in succession. The tissue pieces collected on the 280-mesh sieve were isolated glomeruli that were cultured in Eagle's MEM, supplemented with 20% FCS, with 5% CO₂ in the atmosphere at 37°C. After ~4 wk, the outgrown cells from cultured glomeruli reached confluency and were subcultured using trypsin. Cultured mesangial cells were obtained and maintained by this procedure. All experiments were performed on cells within 15 passages.

Extraction of Proteins from Cultured Mesangial Cells. Mesangial cells were grown to confluency (~10⁷ cells/dish), rinsed with 0.02 M phosphate, 0.15 M NaCl, pH 7.4 (PBS), and scraped from the dishes. The cells were then washed twice with PBS and treated with a buffer containing 8 M urea, 100 mM Tris HCl, 2 M NaCl, 1% β-ME, pH 7.0. About 5 x 10⁷ cells were rotated end-over-end in 1 ml buffer for 3 h at RT. Insoluble material was pelleted and the supernatant was mixed with electrophoresis sample buffer (17), which contained 1% SDS and was subjected to SDS-PAGE.

ELISA. ELISAs were performed as previously described (12) using SM1(19-32)C (0.5 μg/well) as the solid phase antigen. ELISA inhibition tests were performed by incubating a constant dilution (1:200) of antisemur 8652 for 30 min at 37°C with serial dilutions of synthetic peptides before adding to the antigen-coated microtiter wells. The dilution of immune serum used in the inhibition assays gave an absorbance reading at 0.5-0.6 at 450 nm in the absence of inhibiting antigens.

SDS-PAGE and Western Blots. SDS-PAGE was performed in 8% polyacrylamide slab gels according to the method of Laemmli (17) and Western blot analyses were performed as previously described by Towbin et al. (18). Western blot inhibition tests were performed by preincubating the appropriately diluted antisemur with a 100-μM concentration of synthetic peptides (see Results) before adding to the nitrocellulose strips containing the transferred proteins (3).

Analytical Methods. Quantitative amino acid analysis was performed on peptide samples hydrolyzed in constant boiling HCl with an automatic amino acid analyzer (model 121 MB; Beckman Instruments, Inc., Fullerton, CA) (19). Amino acid sequence analysis was performed
with a microsequenator (model 890C; Beckman Instruments, Inc.) according to the principles first described by Edman and Begg (20) and as previously described (19).

**Synthesis of Human Vimentin Peptides.** Peptide copies from two regions of human vimentin (21) containing the tripeptide sequence Arg-Leu-Arg were synthesized by the solid phase method of Merrifield (22), as described (23). The synthetic peptides were cleaved, deblocked with hydrofluoric acid, and purified by gel filtration on a column of Sephadex G10 (Pharmacia Fine Chemicals, Uppsala, Sweden) as previously described (19). The purity and composition of the peptide were assured by HPLC on Ultrasphere ODS2 (Whatman Inc., Clifton, NJ), by quantitative amino acid analysis, and by automated Edman degradation to the penultimate amino acid residue (19). The peptides were synthesized with a COOH-terminal cysteine to enable coupling to a carrier molecule using a bifunctional crosslinking reagent. The peptide copies of regions of human vimentin are designated SV(63-73)C and SV(178-188)C.

**Indirect Immunofluorescence Assays.** Rat mesangial cells and rat skin fibroblasts (CRL 1213; American Type Culture Collection, Rockville, MD) were grown for 2 d on cover slips in Eagle's MEM, supplemented with 20% FCS, with 5% CO2 in the atmosphere at 37°C. The cells on the cover slips were rinsed with PBS and then fixed with 1% paraformaldehyde for 5 min. The cover slips were washed in PBS, incubated with purified crossreactive antibodies at a dilution of 1:4 in PBS for 30 min at room temperature, washed thoroughly with PBS, and incubated with fluorescein-conjugated goat anti-rabbit IgG (CooperBiomedical, Inc., Malvern, PA) at a dilution of 1:40 in PBS for 30 min at room temperature. After washing again, the cover slips were mounted in gelvatol on microscope slides and examined with a fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY). Indirect immunofluorescence tests with frozen sections (4 μm thick) of rat and normal human renal cortex were performed as previously described (1, 24). The specificity of the crossreactive antibodies was tested by immunofluorescence inhibition assays. The immune serum 8652 was preincubated at a dilution of 1:4 in PBS for 30 min at 37°C with synthetic peptides at a concentration of 100 μM or with 0.5 mg/ml of vimentin or desmin, and then used in the indirect immunofluorescence tests as described (1, 24).

**Results**

Previous studies have shown that antisera raised against synthetic peptides of the NH2 terminus of type 1 M protein react with human, mouse, and rat glomeruli (12). The crossreactive epitope was localized to a tetrapeptide sequence Ile-Arg-Leu-Arg at positions 23–26 from the NH2-terminal end of the mature type 1 M protein molecule (12). The purpose of the present study was to identify the autoimmune target in host tissues.

**Crossreactivity with Cultured Mesangial Cells and Fibroblasts.** Since the binding pattern of the crossreactive antibodies in the glomeruli suggested mesangial cell distribution of the antigens, we isolated rat glomeruli and used them to obtain primary cultures of mesangial cells (14). The cells were cultured on cover slips and used in indirect immunofluorescence tests. Antibodies from the strongest crossreactive antiserum 8652 (ELISA titer against Ile, Arg, Leu, Arg, 1:3,200) raised against SM1(1-19) (23–26)C were affinity purified over a column of Affi-Gel 10 to which the synthetic peptide had been covalently linked (12). The purified antibodies (ELISA titer against Ile, Arg, Leu, Arg, 1:1,600) reacted with the mesangial cells in a reticular pattern (Fig. 1 A). The reaction was totally inhibited by preincubation of the antibodies with SM1(1-26)C (Fig. 1 B). Similar results were obtained with antibodies (ELISA titer against Ile, Arg, Leu, Arg, 1:800) purified over an Ile-Arg-Leu-Arg-Cys-Affi-Gel 10 column (data not shown), suggesting that antibodies directed against the COOH-
terminal region of the peptide were responsible for the crossreactions. Incubation of the crossreactive antibodies with rat skin fibroblasts showed a similar fibrillar staining pattern (Fig. 1 C); the reaction was also totally inhibited by preincubation of the antiserum with SM1(1-26)C (Fig. 1 D). The patterns of antibody binding in the two cell types suggested a reaction with a class of protein fibrils known as intermediate filaments or 10-nm filaments.

Identification of Crossreactive Antigen. To identify the crossreactive antigen, cultured mesangial cells were extracted with a buffer containing 8 M urea, 100 mM Tris HCl, 2 M NaCl, 0.1% β-ME, pH 7.0, and the extracted proteins were analyzed by SDS-PAGE (Fig. 2, lane J). For ease of comparison, the two proteins, vimentin and desmin, composing the intermediate filaments of glomerular mesangial cells (25), were purchased commercially (see Materials and Methods) and applied to the same 8% SDS-PAGE (Fig. 2, lanes 2 and 3, respectively). Several protein bands were detected in the mesangial cell extract (Fig. 2, lane J). Three of these reacted with anti-M protein peptide antibodies purified from rabbit antiserum 8652; they migrated as 56-, 52-, and 49-kD polypeptides (Fig. 2, lane J). Furthermore, the antibodies reacted with purified vimentin but not desmin (Fig. 2, lanes 5 and 6). The reaction with
the mesangial extract was completely blocked by absorption of antiserum 8652 with whole M1 but not M24 streptococci (data not shown), indicating the accessibility of the crossreactive epitope on intact M1 protein.

These results suggested that the 56-kD protein is vimentin and the lower molecular weight proteins are degradation products of this protein. It should be mentioned that the purchased vimentin was purified from bovine lens and the desmin was purified from chicken stomach, whereas the proteins in the present study were extracted from rat mesangial cells. The fact that the proteins originate from different species and that the extracted proteins were applied to the gel in 4 M urea, whereas the purified proteins were applied in 0.3 M urea, may account for the slight difference in the apparent molecular weights of bovine vimentin and the 56-kD protein.

To determine whether vimentin could inhibit the renal glomerular crossreactivity of antiserum 8652, immunofluorescence inhibition tests were performed. Bovine vimentin and the synthetic peptide SM1(1–26)C completely inhibited renal glomerular fluorescence, whereas chicken desmin had no inhibitory effect (Table I). Moreover, the crossreactive antibodies were completely absorbed by M1 but not M24 streptococcal cells (Table I). These results indicated that the crossreactive antibodies bind to the native M protein molecule and to similar determinants of authentic vimentin.
and the glomerular crossreactive protein(s), providing additional evidence that the crossreactive antigen is vimentin.

To further confirm this notion, we investigated whether monoclonal and polyclonal antibodies against human vimentin would react with the crossreactive proteins extracted from mesangial cells. Both types of antibodies reacted strongly in Western blots with the three crossreactive proteins extracted from rat mesangial cells (Fig. 3, lanes 2 and 3), whereas an mAb against human desmin did not react with any of the extracted proteins (Fig. 3, lane 4). The mAbs against vimentin reacted with renal glomeruli and mesangial cells in a pattern identical to that of the crossreactive antiserum raised against the type 1 M protein molecule, antiserum 8652 was absorbed twice at ambient temperature for 30 min with pellets collected from 50 ml of an 18-h culture of M type 1 and M type 24 streptococci.

Localization of the Crossreactive Epitope(s) of Vimentin. Since the glomerular crossreactive epitope of type 1 M protein previously was shown to be specified by the tetrapeptide sequence Ile-Arg-Leu-Arg (12), we searched for homologous amino acid sequences in human vimentin (21). We found that vimentin contains the sequence Arg-Leu-Arg, both at position 68-70 and at 183-185. To determine whether these sequences flanked by adjacent sequences of vimentin might be recognized by the crossreactive antibodies, we synthesized two 11-residue peptides encompassing the two Arg-Leu-Arg sequences and tested their crossreactivity in immunofluorescence and Western blot inhibition assays. The synthetic peptides, designated SV(63-73)C and SV(178-188)C, both totally inhibited the glomerular crossreactivity of antiserum 8652 (Table II). As previously reported (12), and as shown again in Table II for comparison, peptides of M1 protein containing the sequence Ile-Arg-Leu-Arg, but not those lacking this sequence, similarly blocked immunofluorescence.

These results were confirmed in Western blot and quantitative ELISA inhibition tests. Both synthetic peptides of vimentin encompassing the sequence Arg-Leu-Arg blocked the reaction of antiserum 8652 in Western blots with the urea-extracted proteins of mesangial cells as well as with authentic bovine vimentin (data not shown). In ELISA inhibition tests of antiserum 8652 against the M protein peptide SM1(19-
FIGURE 3. Western blot analysis of the reactivity of urea-extracted mesangial proteins with antisera to vimentin and desmin. (Lane 1) Coomassie blue-stained SDS-gel of urea-extracted mesangial proteins. Western blots (lanes 2-4) of the same sample showed reaction of 56-, 52-, and 49-kD proteins with an mAb to human vimentin (lane 2), with polyclonal antibodies to human vimentin (lane 3), but not with an mAb to human desmin (lane 4). All sera were tested at a 1:200 dilution.

32)C, both vimentin peptides were inhibitory in a dose-related fashion (Fig. 4). On a molar basis, however, the vimentin peptide SV(63-73)C was ~100-fold more inhibitory than SV(178-188)C and was equal in potency to the M protein pentapeptide Ile-Arg-Leu-Arg-Cys. We chose peptide SM1(19-32)C as the ELISA antigen.

Table II

| Inhibitory peptide* | Amino acid sequence† | Renal glomerular fluorescence |
|---------------------|----------------------|-----------------------------|
| None                |                      | + + +                       |
| SV(63-73)C          | RSSAVRLSSVC         | −                           |
| SV(178-188)C        | AEDIMRLREKLC        | −                           |
| SM1(19-32)C         | AIQNIRLRENKDLC      | −                           |
| SM1(23-26)C         | IRLRC               | −                           |
| SM1(1-12)C          | NGDGNPREVEDC        | + + +                       |

* Each synthetic peptide was tested at a concentration of 100 μM, except for IRLRC, which was tested at a concentration of 5 mM.

† The sequence of the NH2-terminal region of type 1 M protein was confirmed in a previous study (13). The human vimentin sequence was derived from a cDNA clone (21). The single letter code for amino acid residues is used to conserve space and for ease of comparison.

§ Underscored cysteine residues were added for the purpose of coupling to a carrier.

¶ These data have been reported previously (12) and are shown again here for comparison.
Figure 4. Inhibition of ELISA reactions of antiserum 8652 with immobilized SM1(19-32)C by synthetic peptides. A constant dilution (1:200) of anti-SM1(1-19) (23-26)C was incubated with increasing concentrations of SM1(19-32)C (A), SV(63-73)C ( ), SV(178-188)C ( ), SM1(23-26)C ( ), and SM1(1-12)C (x) as soluble inhibitors. The highest concentration of synthetic peptides was 500 μM and the lowest concentration was 0.0064 μM, reached by serial fivefold dilutions.

Discussion

In this study, we have shown that an antiserum raised against an NH2-terminal peptide of type 1 M protein crossreacts with vimentin, a component of the cytoskeleton of eukaryotic cells. The cytoskeleton of most eukaryotic cells is composed of three major fiber systems: the microfilaments (5–6 nm in diameter), the microtubules (20–25 nm in diameter), and the intermediate filaments (7–11 nm in diameter). Five major classes of intermediate filaments have been described, reviewed by Lazarides (26, 27): the cytokeratins in epithelial cells, the neurofilaments in neurons, the glial filaments in astrocytes, desmin in myogenic cells, and vimentin in various nonepithelial cells, in particular, mesenchymal cells. The subunits of each of the intermediate filament proteins have common structural features, reviewed by Steinert and Parry (28). The subunit is composed of a central α helix-rich (rod) domain of ~311–314 amino acid residues, and NH2-terminal (head) and COOH-terminal (tail) domains of variable size and chemical character. Generally, neither of the latter assume an α-helical conformation. The α-helical domains of these proteins give rise to a coiled-coil fibril with the amino acid sequences following a seven-residue periodicity of the form a-b-c-d-e-f-g, in which residues at positions a and d are usually apolar and those at g are usually polar. This repeating pattern of residues gives rise to a coiled-coil fibril with the apolar residues buried along its axis.

Each of the M proteins of group A streptococci studied thus far shows a similar seven-residue periodicity of polar and apolar amino acids (13, 29, 30). In addition, it has been shown that the M proteins protrude as α-helical coiled-coil fibrils from the surface of virulent strains of streptococci (9). These fibrils render the organisms resistant to recognition and ingestion by phagocytes of the nonimmune host (8, 10). The similarity of the M protein α-helical structure to that of mammalian coiled-coil proteins, such as myosin, keratin, vimentin, desmin, and fibrinogen, was pointed
out earlier by Manjula et al. (30). It should not be surprising, therefore, that the only M protein crossreactive tissue proteins identified thus far belong to this group of proteins.

Krisher and Cunningham (5) first described a crossreaction of mAbs to type 5 streptococci with cardiac myosin. Later it was shown that types 5, 6, and 19 M proteins contain epitopes that are shared with cardiac myosin (3, 4). Most recently, we identified a renal glomerular crossreactive tetrapeptide sequence, Ile-Arg-Leu-Arg, in the NH$_2$ terminus of type 1 M protein (12). Because the staining pattern of the crossreactive antibodies suggested a mesangial cell distribution of the antigen in the glomeruli, we undertook the present study of the crossreactivity of these antibodies with cultured mesangial cells.

The staining pattern obtained with mesangial cells and fibroblasts suggested that intermediate filaments were involved in the crossreactions. Since only two intermediate filaments, desmin and vimentin, had been found in glomerular mesangial cells and fibroblasts (25, 26, 31), we focused our investigations on these two proteins and found vimentin to be the autoimmune target. The evidence that vimentin is the target is as follows: (a) the M protein peptide crossreactive antibodies reacted with authentic vimentin but not desmin in Western blots; (b) vimentin, but not desmin, inhibited the crossreactions both in immunofluorescence and Western blot tests; (c) monoclonal and polyclonal antibodies raised against human vimentin reacted with the putative vimentin extracted from rat mesangial cells in the same pattern as that obtained with the crossreactive antiserum; (d) the mAbs against human vimentin reacted with glomeruli and mesangial cells in a pattern identical to that of the crossreactive antibodies; and (e) synthetic peptides copying vimentin sequences encompassing the tripeptide Arg-Leu-Arg inhibited the reaction of the M1 protein peptide antiserum with mesangial cells and extracted vimentin.

Extensive homologies between the sequences of vimentins of different species have been reported. The sequence of human vimentin (21) is 98% homologous to that of Syrian hamster vimentin (32), and the hamster sequence exhibits 80% homology with that of chicken vimentin (33). The high degree of homology between the two mammalian vimentin sequences suggests that the sequence of rat vimentin is similarly homologous. It is probable that the 56-kD protein, which is similar in molecular mass to that of human vimentin, represents rat vimentin and the lower molecular weight proteins stained with the monoclonal antivimentin antibody represent degradation products of this protein. Such degradation of vimentin had been noticed in earlier studies by Franke et al. (34).

Because the crossreactive epitope of type 1 M protein was previously shown to be specified by the tetrapeptide sequence Ile-Arg-Leu-Arg (12), we searched for homologies in the amino acid sequence of human vimentin (21). Two regions were found containing the tripeptide sequence Arg-Leu-Arg at positions 68–70 and 183–185. Region 67–70 is part of the random-coil NH$_2$-terminal (head) domain, whereas region 182–185 lies in the $\alpha$-helical (rod) domain of the vimentin sequence. By comparison, the crossreactive epitope Ile-Arg-Leu-Arg at positions 23–26 of type 1 M protein is predicted to be located at the beginning of the $\alpha$-helical coiled-coil structure of the molecule. Taking into account the primary and secondary structural features of the potentially crossreactive epitopes in human vimentin and type 1 M protein, one might predict that the sequence Arg-Leu-Arg at positions 183–185 in the
α-helical part of vimentin would be the major binding site of the crossreactive antibodies. However, the two 11-residue synthetic peptides of vimentin encompassing each of the Arg-Leu-Arg sequences and adjacent amino acids exhibited markedly different potencies in their inhibitory activities against the crossreactive antiserum 8652; the peptide SV(63–73)C was ~100-fold more inhibitory than SV(178–188)C. We cannot conclude from these results which of these sites is more accessible in the intact vimentin molecule because tertiary structural constraints undoubtedly play a role (35).

In this regard, the crossreactive antibodies did not react with chicken desmin, although this protein contains an amino acid sequence, Arg-Leu-Arg, at positions 132–135 (36). Either this sequence is inaccessible in the intact molecule or it does not by itself represent an epitope that is recognized by antiserum 8652; sequence identity of short peptides does not ensure similarity of structural shape (35).

In summary, we have demonstrated that antibodies directed against the epitope Ile-Arg-Leu-Arg of the nephritogenic type 1 M protein react with the intermediate filament protein vimentin of renal glomerular mesangial cells. The identification of the crossreactive antigen is one important step toward establishing a connection between autoimmune reactions and the pathogenesis of acute poststreptococcal nephritis. Further studies are in progress to assess the accessibility of mesangial cell vimentin to the crossreactive antibodies and to determine the role of autoimmune reactions to streptococcal M proteins in the generation of acute and chronic inflammatory diseases.

Summary

The crossreactivity of antibodies against a renal autoimmune epitope of Streptococcus pyogenes M protein with glomerular mesangial cells was investigated. The antibodies directed against the amino acid sequence Ile-Arg-Leu-Arg of the nephritogenic type 1 M protein reacted in a fibrillar pattern with mesangial cells cultured from isolated glomeruli. In Western blots of urea-extracted mesangial proteins, the antibodies reacted with a 56-kD protein. Monoclonal and polyclonal antibodies identified the 56-kD mesangial protein as vimentin. Two synthetic peptides of human vimentin containing the sequence Arg-Leu-Arg reacted with the autoimmune antibodies raised against a streptococcal M protein peptide. These results provide evidence that the intermediate filament protein vimentin shares autoimmune epitopes with streptococcal M protein.

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