POLYSPECIFICITY OF ANTISTREPTOCOCCAL MURINE MONOCLONAL ANTIBODIES AND THEIR IMPLICATIONS IN AUTOIMMUNITY

By MADELEINE W. CUNNINGHAM and ROBERT A. SWERLICK

From the University of Oklahoma Health Sciences Center, Department of Microbiology and Immunology, and Department of Dermatology, Oklahoma City, Oklahoma 73190

Patients with acute rheumatic fever (ARF)[1] have been shown to have circulating heart-reactive antibodies (HRA), often in high titer, and examination of the myocardium of some of these patients at autopsy has revealed the presence of bound antibody and complement (1, 2). HRA in ARF have been shown by van de Rijn et al. (4) to react with streptococcal membrane polypeptides, and they have also been generated in rabbits immunized with group A streptococcal membranes (3, 4). However, studies by Kaplan (5) have previously implicated cell wall components and M protein of group A streptococci in the immunologic crossreactions between HRA and streptococcal antigens. Since M protein is the major virulence determinant of group A streptococci, and since antibodies to the M protein protect against infection with homologous type streptococci, the production of a safe vaccine has been the purpose of innumerable studies of the antigenic composition of M proteins and their immunologic crossreactivity with heart tissue (5–9). Analysis of homogeneous M protein preparations by Dale and Beachey (6) revealed that M types 5 and 19 possess heart crossreactive epitopes, and Poirer et al. (9) found that the cloned type 5 M protein gene produced M protein that retained the epitopes that bind HRA. Type 1 M protein has also been implicated in crossreactions with HRA (7, 8).

The results of these past studies support the hypothesis that at least some of the M proteins, especially M type 5, share determinants with host antigens. Furthermore, anti–M type 5 antibodies were shown to react with a high molecular weight sarcolemmal antigen (6), and structural studies of type 5 M protein by Fischetti and Manjula (10, 11) have shown its structure to be similar to both tropomyosin and myosin.

Other studies to determine the capacity of antistreptococcal sera to react with host tissues have shown that these antisera react not only with heart tissue but also with skin (8), brain (12), skeletal muscle (3), and thymus (8). Fibroblasts have also been shown (13) to absorb HRA from ARF sera, and streptococcal membranes absorbed the antibodies that reacted with the fibroblasts. However, until recently, the autoantigens involved in these crossreactions were unidentified.

Our studies have primarily focused on the use of anti–*Streptococcus pyogenes*

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[1] *Abbreviations used in this paper:* ARF, acute rheumatic fever; HRA, heart-reactive antibodies.

mAbs as probes for the crossreactive antigens shared between the streptococcus and human heart tissue. The reaction of these mAbs with heart tissue proteins revealed a high molecular weight antigen that was subsequently identified as myosin (14). In a study of the fragments of myosin, it was shown that crossreactive sites resided in either the light or heavy meromyosin subfragments of the heavy chain. In addition, the mAbs were found to be specific for either skeletal myosin alone or for both cardiac and skeletal myosin (15).

In this report, the mAbs that react with group A streptococci and myosin were used as probes to identify additional crossreactive antigens in host tissues. One of the streptococcal mAbs reacted with myosin, DNA, and synthetic nucleotide homopolymers, and another crossreactive mAb was specific for myosin, keratin, and actin, a family for α helical coiled-coil proteins.

The streptococcal antigens reactive with these antibodies in Western immunoblots were streptococcal membrane protein(s) and pep M5 protein extracts. The present study describes the reactions of these crossreactive mAbs with both streptococcal antigens and a variety of autoantigens, and we discuss the implications of these findings.

Materials and Methods

Streptococci. M type 5 *Streptococcus pyogenes* (*manfraedo*) was obtained from Edwin H. Beachey, Veterans Administration Medical Center, Memphis, TN. Streptococci were cultured at 37°C in chemically defined medium (CDM), according to the method of van de Rijn and Kessler (16).

Streptococcal Membranes. Membranes were purified from whole streptococci broken with sterile glass beads in a Bronwill cell homogenizer (Bronwill Scientific, Inc., Rochester, NY [17]). Purification included differential centrifugation with a final centrifugation at 100,000 *g*, as previously described (18).

Chemicals. Poly(A), Poly(C), Poly(U), Poly(I), Poly(dT), and Poly(dA) were from P-L Biochemicals, Inc., Milwaukee, Wisconsin. Calf thymus DNA and histones were obtained from Calbiochem-Behring Corp., La Jolla, California. BSA (fatty acid– and globulin-free), egg white lysozyme, human epidermal keratin, rabbit heart actin, beef heart cardiolipin, rabbit skeletal muscle myosin, PC, and human and mouse IgG were from Sigma Chemical Co., (St. Louis, MO) and calf skin collagen was from Worthington Biochemical Corp., Freehold, NJ.

Pepsin-extracted M Protein. ~30 grams of group A, M type 5 streptococci, cultured in CDM and washed twice in PBS, were treated with pepsin at pH 5.8 according to the method of Manjula and Fischetti (19). Streptococci were washed twice in sodium phosphate buffer, pH 5.8, and were suspended at a concentration of 1 g of organisms per 2 ml of buffer. The cell suspension was warmed to 37°C and pepsin was added at a concentration of 1 mg per 10 g of bacteria. Digestion was performed at 37°C with slow stirring, and after 45 min pepsin activity was neutralized by adding sodium bicarbonate to raise the pH to 7.4 at 0°C. The suspension was centrifuged at 15,000 rpm for 15 min, and the supernatant was collected. The streptococci were resuspended in phosphate buffer, pH 5.8, and the digestion procedure was repeated. Supernatants from the first and second digestions were sterilized by filtration through a 0.22 μm Millipore filter and were kept separated. The supernatants were concentrated by ultrafiltration through a PM10 membrane (Amicon Corp., Danvers, MA), were stored at 4°C, and were tested for reactivity with anti–*S. pyogenes* M type 5 sera kindly provided by Dr. Richard Facklam (Centers for Disease Control, Atlanta, GA.). Purity of the preparations was determined by SDS-PAGE and Western immunoblotting.

Monoclonal Antibodies. Murine hybridoma cell lines were selected, cloned, stored and maintained as previously described (20). mAbs used in this study included 36.2.2, 49.8.9,

and 54.2.8, which were stored as culture fluids at 4°C. Characteristics of these mAbs have been reported elsewhere (14, 18, 20).

ELISA, Competitive Inhibition Assays, and Western Immunoblotting. The ELISA was performed according to methods previously described (20). The ELISA was used to determine reactions of the mAbs with M type 5 *S. pyogenes*, glutaraldehyde-fixed to polyvinyl chloride microtiter plates. In competitive inhibition assays, which have been previously described (15), antibodies were mixed 1:1 with increasing concentrations of inhibitor and incubated 1 h at 37°C and overnight at 4°C. The antibody was then mixed in duplicate with M type 5 *S. pyogenes* attached to microtiter plates, and the ELISA was performed as previously described (20).

Western immunoblots were performed according to the method of Towbin et al. (21) and PAGE was performed in gradient gels according to the method of Laemmli (22). 10–20% slab gel gradients were prepared from an acrylamide stock solution containing 30 g acrylamide (Sigma Chemical Co.) in 100 ml distilled water. Gradients were prepared by mixing solutions in a gradient maker. The 10% acrylamide solution contained 6.66 ml of the acrylamide stock solution, 0.4 ml glycerol, 2.5 ml of 1.5 M Tris-HCl buffer (pH 8.9), 9.96 ml distilled water, 0.2 ml 10% SDS, 0.008 ml TEMED, and 0.135 ml freshly prepared ammonium persulfate. The 20% acrylamide solution was prepared by mixing 13.34 ml of the acrylamide stock solution with 2.4 ml glycerol, 2.5 ml of 1.5 M Tris-HCl buffer (pH 8.9), 0.46 ml distilled water, 0.20 ml 10% SDS, 0.002 ml TEMED, and 0.05 ml freshly prepared 10% ammonium persulfate. The stacking gel was prepared by mixing 1.5 ml of acrylamide stock solution with 1.25 ml of 0.5 M Tris-HCl buffer (pH 6.8), 7.10 ml distilled water, 0.10 ml 10% SDS, 0.005 ml TEMED, and 0.1 ml of freshly prepared 10% ammonium persulfate.

Before loading onto polyacrylamide gels, samples were boiled for 3–5 min in 1–5% SDS and 10% 2-ME in Tris buffer (pH 7.2) containing glycerol and bromphenol blue tracking dye. Samples were separated by electrophoresis for 4–6 h at 35 mA per gel slab and then were electroblotted against nitrocellulose membranes (0.45 μm) for 18 h at 80 mA. A portion of each blot was stained for protein with amido black in acetic acid and methanol, as previously described (18). Culture fluids containing mAbs were mixed with the other portions of the blot, and antibody reactivity was detected by peroxidase-conjugated anti-mouse IgM (Cappel Laboratories, Cochranville, PA) and subsequent development in the substrate H_2O_2 and chloronaphthol, as described before (18). Blots were dried and photographed. Molecular weights of proteins were estimated using molecular weight marker standards (Sigma Chemical Co.)

Human Cell Lines and Cultures. Human skin fibroblasts were obtained from the skin of normal human volunteers. The dermis obtained from punch biopsy specimens was dissected free of epidermis and plated into six-well tissue culture plates (Costar, Cambridge, MA) with DMEM plus 10% FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM L-glutamine. Fibroblasts grew preferentially and were identified by their morphology and staining leucine aminopeptidase activity (23). The A431 squamous carcinoma and the A549 lung carcinoma cell lines were obtained from American Type Culture Collection (Rockville, MD) and were maintained in DMEM with 10% FCS, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and 2 mM L-glutamine. Cells were grown to confluence on sterile coverslips, washed in PBS, fixed in cold acetone, dried and stored dessicated at −20°C until used.

Immunofluorescence Staining of Cell Lines. Cells fixed on coverslips were rehydrated in PBS, and covered with 200 μl of culture fluid containing the appropriate mAb. After a 30 min incubation at room temperature, the coverslips were then washed in PBS, and 200 μl of fluorescein-conjugated anti-mouse IgM (Cappel Laboratories) diluted 1:15 in PBS was applied to each coverslip and incubated at room temperature for 30 min. The coverslips were again washed in PBS, mounted on glass slides with PBS-glycerin, and read on a Laborlux microscope (E. Leitz, Rockleigh, NJ) equipped with epifluorescence. A549 cells were examined for the presence of keratin intermediate filaments by incubation of fixed cells with guinea pig anti-cow prekeratin antisera (Miles Laboratories, Inc., Naperville, IL) diluted 1:10 in PBS for 30 min at room temperature followed by fluorescein-

conjugated goat anti-guinea pig Igs (U. S. Biochemical Corp., Cleveland, OH). As positive and negative controls, A431 cells and human fibroblasts were examined using anti-prekeratin antisera.

Antibody Blocking Studies Using Anti-Vimentin Antibodies. 200 μl of rabbit anti-human vimentin antisera (Transformation Research, Cambridge, MA.), diluted 1:5 in PBS, was placed on coverslips containing fixed human fibroblasts, and was incubated for 30 min at room temperature. After three washes in PBS, the coverslips were incubated with mAb 54.2.8 for 30 min, washed in PBS, and then incubated with fluorescein-labeled goat anti-rabbit Ig (U. S. Biochemical Corp.). After a 30 min incubation at room temperature, coverslips were again washed in PBS, mounted in PBS/glycerin, and examined for fluorescence.

Results

Antistreptococcal Activity of mAbs. Mouse hybridomas secreting antibodies that bind to group A streptococci were selected as previously described (20). Two (36.2.2 and 54.2.8) of this group of hybridomas producing antibodies crossreactive with heart and streptococci were found to react with myosin (14). One mAb (49.8.9) that did not react with myosin but was crossreactive with human heart extracts has continued to be studied and is included herein. All three mAbs antibodies are the IgM isotype.

We tested the mAbs for binding to streptococcal antigens in the Western immunoblot. Whole group A, M type 5 streptococci and their membranes were extracted with 5% SDS, and the supernatants containing streptococcal antigens were separated by PAGE and were electroblotted onto nitrocellulose membranes. The blots were subsequently reacted with the mAbs and a normal mouse IgM (20 μg/ml) control. Fig. 1 illustrates the results of this experiment. mAbs 36.2.2, 49.8.9, and 54.2.8 all reacted with several streptococcal antigen(s) of high molecular weight and with a triplet of bands near 58,000. Purified M type 5 streptococcal membranes were also treated with 5% SDS and compared with the whole cell extracts. mAbs 36.2.2 and 54.2.8 reacted strongly with a membrane antigen near 62,000–67,000. These results demonstrate that the antibodies reacted simultaneously with components from whole cells and membranes.

Since the triplet of bands in streptococcal whole cell extracts corresponded to the molecular size of M protein, pepsin extracts of M type 5 *S. pyogenes* were separated by electrophoresis and electroblotted onto nitrocellulose. The protein from the first extraction contained only peptides of type 5 M protein based on their positive reaction with M type 5–specific antisera (a generous gift of Dr. Richard Facklam, Centers for Disease Control). The larger fragment of the pep M 5 protein preparation reacted with mAbs 36.2.2 and 54.2.8 in the Western immunoblot (Fig. 2). The reaction of these mAbs with a similar sized band was seen when purified pep M 5 (obtained as a generous gift from Dr. Edwin Beachey, Veterans Administration Medical Center, Memphis, TN) was used as antigen. To test another type of M protein, cloned M 6 protein from *S. pyogenes* was obtained as a generous gift from Dr. Vincent Fischetti (The Rockefeller University). Both pep M 5 and the cloned M 6 proteins were attached to ELISA plates, and mAbs 36.2.2, 54.2.8, and 49.8.9 were mixed with both M types. mAbs 25.3.10 and 25.5.3 (IgM isotype) were included as control antibodies that react with group A streptococci but are essentially unreactive with the M protein. In the ELISA, mAbs 36.2.2 and 54.2.8 reacted with both pep M 5 and the cloned

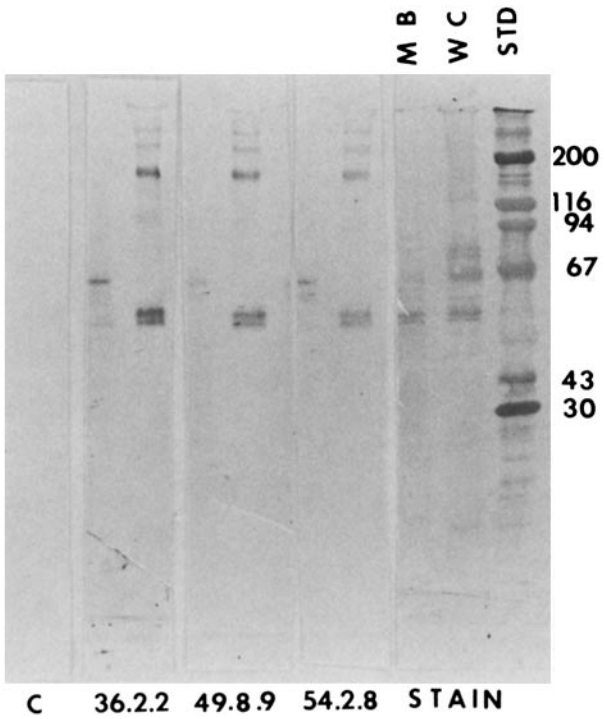

FIGURE 1. Western immunoblot of SDS-extracted, M type 5 *S. pyogenes* membranes (*MB* or *left* lane) and whole cells (*WC* or *right* lanes) after separation in a 10–20% slab gel gradient by SDS-PAGE. Electroblotted MB and WC proteins were mixed separately with amido black (*STAIN*) mAbs 36.2.2, 49.8.9, 54.2.8, and a medium control containing mouse IgM (18 μg/ml). Stained molecular mass markers (× 10^{-3}) are shown (*STD*) for: myosin, 200; β-galactosidase, 116; phosphorylase B, 94; BSA, 67; egg albumin, 43; and carbonic anhydrase, 30.

M 6 proteins, while 49.8.9 reacted only with the pep M 5 (Table I). None of these antibodies was opsonic for M type 5 *S. pyogenes* (data not shown). The capacity of 49.8.9 to react with pep M 5 in the ELISA, but not in the Western immunoblot, suggests that the epitope is labile to SDS treatment.

Evaluation of the Binding of mAbs to Human Cell Lines by Indirect Immunofluorescence. Since fibroblasts were previously shown (13) to absorb HRA from ARF sera, human skin fibroblasts were mixed with mAbs 36.2.2, 49.8.9, and 54.2.8 in an attempt to examine their crossreactive antigens. All three of the mAbs reacted distinctly with the fibroblasts (Fig. 3). mAb 36.2.2 reacted with cytoplasmic cytoskeletal elements, as did 49.8.9 (Fig. 3, *a* and *b*). However, 54.2.8 reacted strongly with both the nucleus and cytoskeletal structures in the cytoplasm (Fig. 3*c*). Similar nuclear fluorescence was observed when 54.2.8 was reacted with A431 squamous carcinoma line (Fig. 4*a*), but filamentous cytoplasmic staining was not prominent when these cells were reacted with any of the mAbs. The A549 tumor cell line, an epithelioid cell line derived from a human lung carcinoma, also showed nuclear staining upon reaction with 54.2.8, and cytoplasmic filamentous staining upon reaction with 49.8.9, but the most striking reactivity was noted with mAb 36.2.2 (Fig. 4*c*). No nuclear fluorescence

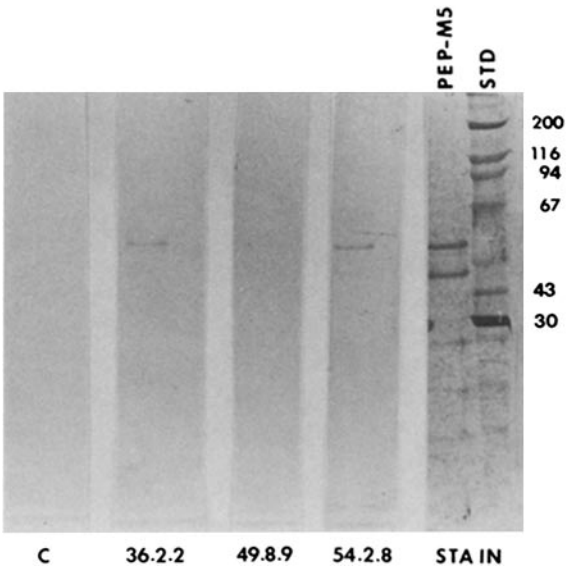

FIGURE 2. Western immunoblot of M type 5 protein extracted with pepsin (Pep M-5) after separation in a 10–20% slab gel gradient by SDS-PAGE. Pep M-5 protein was mixed separately with amido black (*STAIN*) mAbs 36.2.2, 49.8.9, and 54.2.8, and a medium control containing mouse IgM (18 μg/ml). Stained molecular mass markers ($\times 10^{-3}$) are shown (*STD*) for: myosin, 200; β-galactosidase, 116; phosphorylase B, 94; BSA, 67; egg albumin, 43; and carbonic anhydrase, 30.

TABLE I

Comparison of the Binding of Heart-crossreactive mAbs with Pep M 5 and Cloned M 6 Proteins in ELISA

Hybridoma clone	OD_{405}	
	Pep M-5	Cloned M-6
36.2.2	1.819	1.378
49.8.9	1.076	0.291
54.2.8	1.034	1.417
25.5.1.4	0.287	0.118
25.3.1.9	0.269	0.150
Medium control	0.017	0.024
PBS control	0.000	0.007

The cloned M6 was a generous gift from Dr. Vincent A. Fischetti, The Rockefeller University, New York.

was seen, but cytoskeletal elements were brightly labeled. No fluorescence was seen with fibroblasts (Fig. 3*d*), A549 (Fig. 4*d*), or A431 (Fig. 4*b*) cells when they were mixed with normal mouse IgM (10 μg/ml) control medium. Both A431 and A549 cell lines stained positively with anti-keratin serum, but fibroblasts did not (data not shown).

Since fibroblasts contain vimentin as a major cytoskeletal element in the cytoplasm, mAb 54.2.8 was challenged with rabbit anti-vimentin sera for binding of the fibroblast cytoskeleton. Fig. 5*a* shows that normal rabbit sera did not affect the reaction of 54.2.8 with the fibroblasts, whereas anti-vimentin sera

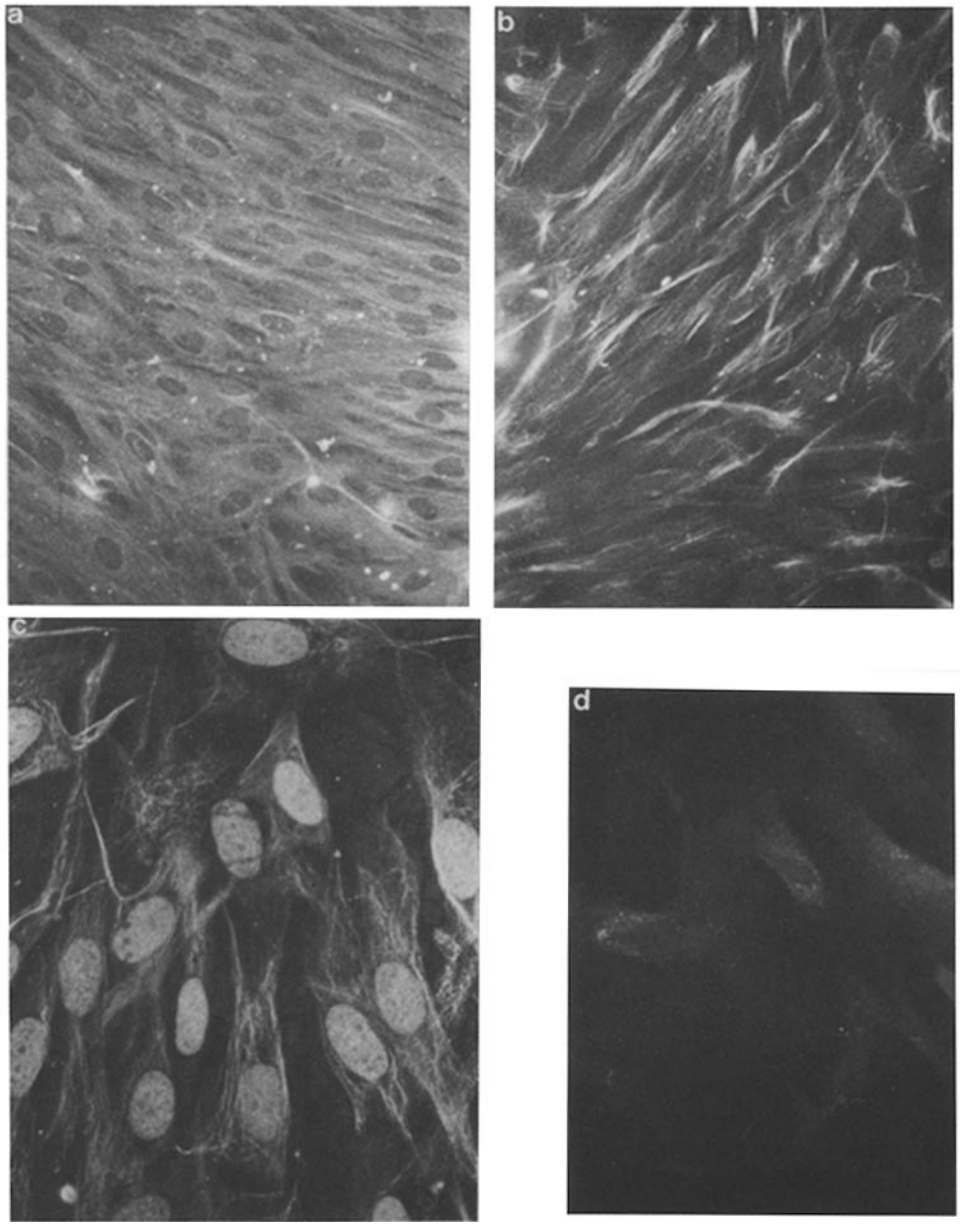

FIGURE 3. Indirect immunofluorescence after the reaction of mAb 36.2.2 (*a*), 49.8.9 (*b*), 54.2.8 (*c*), and medium plus mouse IgM (10 μg/ml) (*d*) with cultured normal human fibroblasts. Original magnification, × 150.

clearly blocked the reaction of 54.2.8 with the cytoplasm while leaving the nuclear staining unchanged (Fig. 5*b*). Disruption of fibroblasts in PBS containing proteinase inhibitors released proteins in the 55,000–65,000 range, near the molecular weight of vimentin (58,000), that reacted with mAb 54.2.8 in Western immunoblots (data not shown).

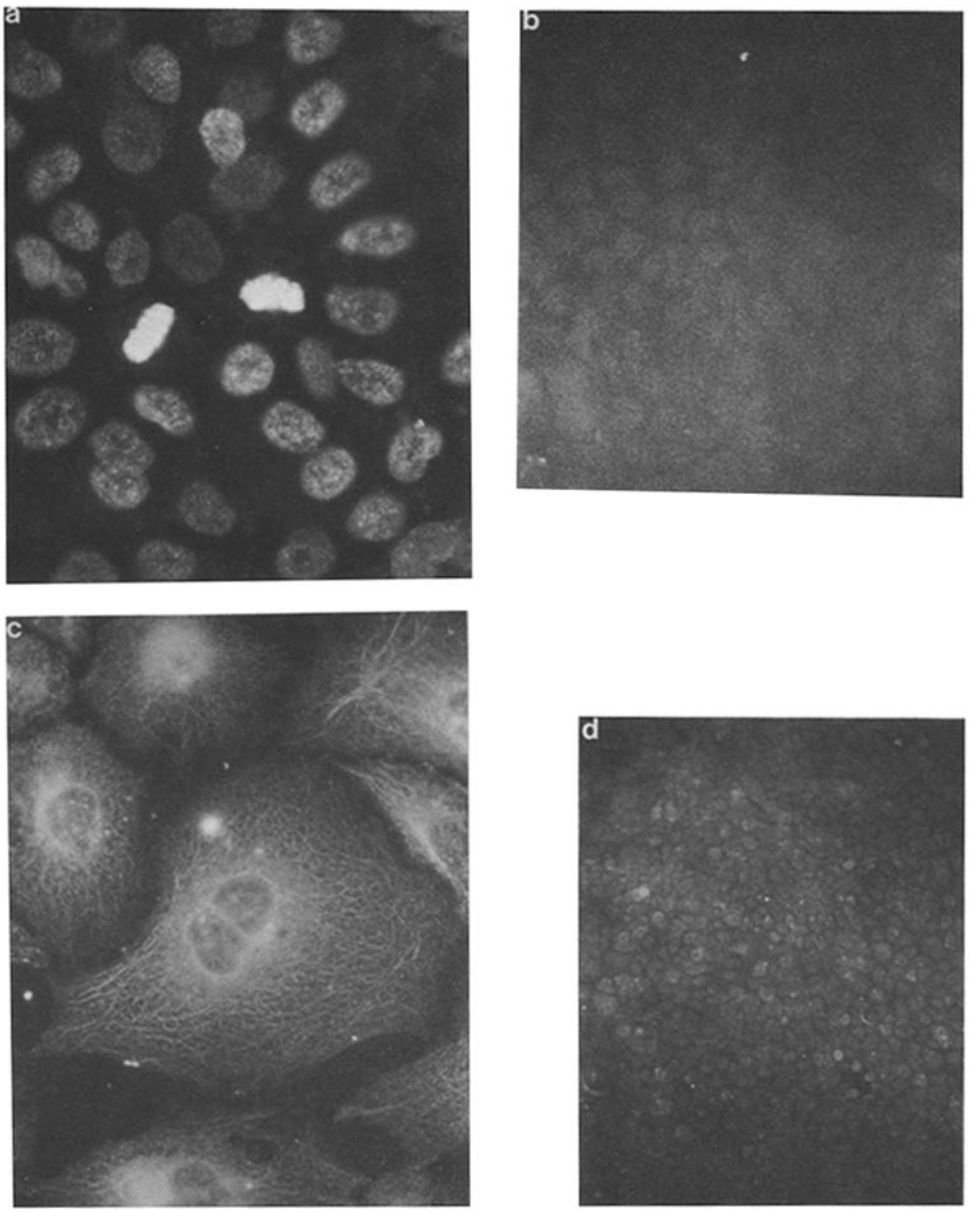

FIGURE 4. Indirect immunofluorescence after the reaction of mAb 54.2.8 (*a*) and control medium containing normal mouse IgM (10 μg/ml) (*b*) with the cultured A431 cell line (original magnification × 100 and × 150, respectively). Reaction of mAb 36.2.2 (*c*) and normal mouse IgM in control medium (*d*) with the cultured A549 cell line (original magnification, × 250 and × 50, respectively).

Competitive Inhibition of mAbs by Multiple Autoantigens. Since mAb 54.2.8 reacted with the nucleus of fibroblasts, it was important to determine whether or not the reaction was, in fact, with DNA or with nuclear proteins or histones. We tested cardiolipin, PC, myosin, and BSA, in addition to calf thymus DNA,

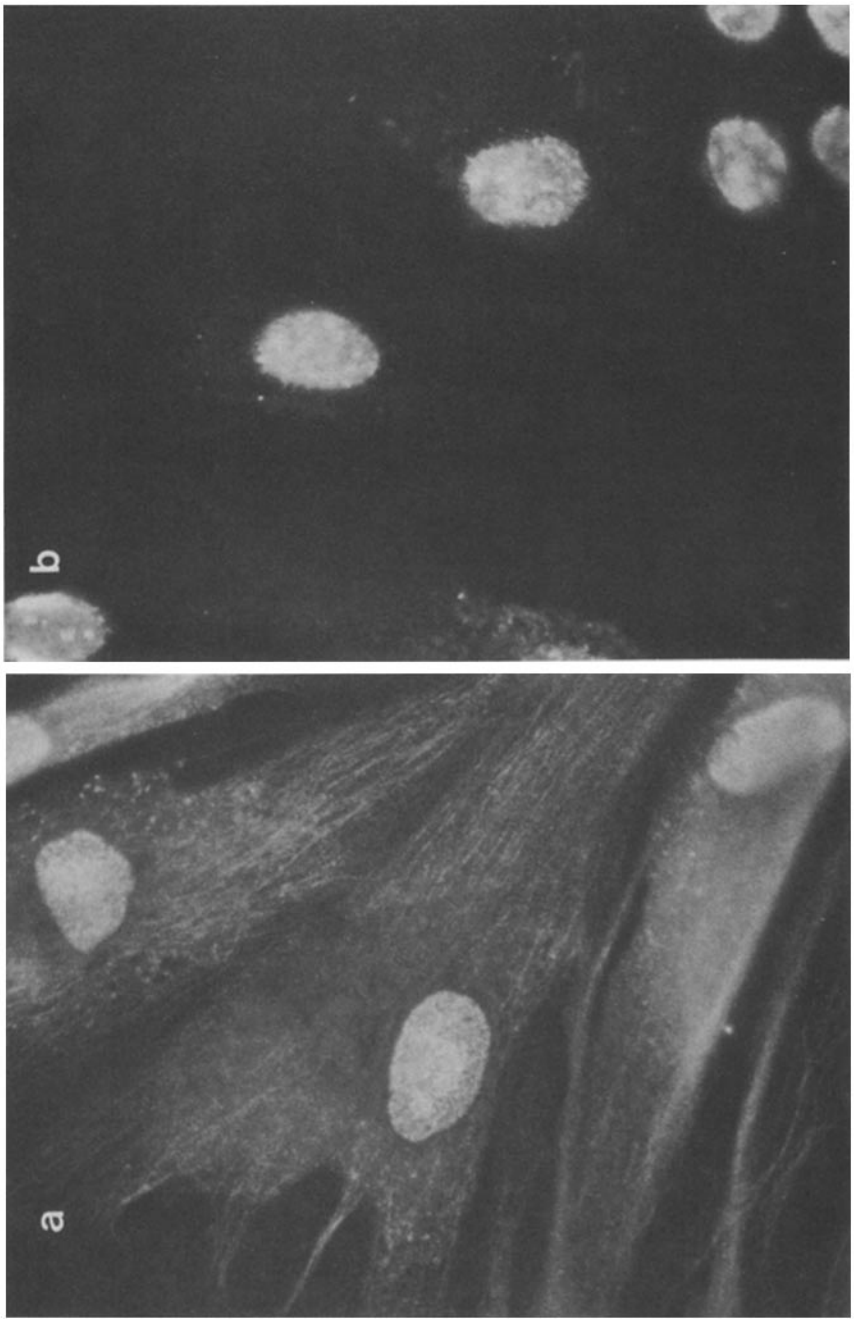

FIGURE 5. Blocking of mAb 54.2.8 with anti-vimentin antibody. Indirect immunofluorescence of human fibroblasts after reaction with mAb 54.2.8 blocked with (*a*) normal guinea pig sera and (*b*) guinea pig anti-vimentin sera (× 250).

TABLE II

Competitive Inhibition of mAbs 36.2.2, 49.8.9, and 54.2.8 by Calf Thymus DNA, Synthetic Polynucleotides, and Myosin

Inhibitor	Concentration (μg/ml) of mAb:*		
	36.2.2	49.8.9	54.2.8
Calf thymus DNA	>500	>500	3.9
Poly(A)	ND	ND	>500
Poly(C)	ND	ND	>500
Poly(U)	ND	ND	500
Poly(I)	ND	ND	7
Poly(dA)	ND	ND	>500
Poly(dT)	ND	ND	87
Myosin	31	>500	15
BSA	>500	>500	>500
Histones	>500	500	>500
Cardiolipin	250	>500	62–125
Phosphoryl choline	>500	>500	>500

* Concentration of mAb producing 50% inhibition of the antibody reactivity with M type 5 group A streptococci in ELISA (A_{405}). Concentrations >250–500 μg/ml were not tested.

TABLE III

Competitive Inhibition of mAb 36.2.2, 49.8.9 and 54.2.8 by a Number of Coiled-coiled Proteins and IgG

Inhibitor	Concentration (μg/ml) mAb:*		
	36.2.2	49.8.9	54.2.8
Myosin	31	>500	15
Keratin	0.97	>500	250
Actin	0.18	>500	500
Collagen	>500	>500	>500
BSA	>500	>500	>500
Lysozyme	>500	>500	>500
Human IgG	>500	500	>500
Mouse IgG	>500	>500	>500

* Concentration of mAb producing 50% inhibition of the antibody reactivity with M type 5 group A streptococci in the ELISA (A_{405}). Concentrations >250–500 μg/ml were not tested.

synthetic nucleotide homopolymers, poly(A), poly(C), poly(U), poly(I), poly(dT), poly(dA), and histones in competitive inhibition assays (Table II). mAbs were incubated with each inhibitor and then mixed in the ELISA with group A, M type 5 streptococci. Calf thymus DNA was a strong inhibitor of mAb 54.2.8 as was poly(I) and myosin. Poly(dT) was inhibitory at 87 μg/ml, and cardiolipin was less inhibitory at ≥125 μg/ml. mAbs 36.2.2 and 49.8.9 were unaffected by DNA and did not react with the synthetic homopolymers. Myosin as the positive control inhibited antibodies 36.2.2 and 54.2.8 as expected, and BSA served as a negative control. Histones and PC did not react with any of the mAbs.

The mAbs were mixed with α helical coiled-coil proteins and IgG (Table III). Myosin was used as the positive control, while BSA and lysozyme were negative

controls. IgG from both mouse and human was tested in the competitive inhibition assays to determine the presence of rheumatoid factor antibodies. Keratin, actin, and collagen were also tested for reactivity with the crossreactive mAbs. Both keratin and actin strongly inhibited mAb 36.2.2, but they had little affect on antibodies 49.8.9 and 54.2.8. Collagen failed to inhibit any of the antibodies. None of the crossreactive antibodies were rheumatoid factors since they were not inhibited with mouse or human IgG. BSA and lysozyme had no affect as negative controls. The results show that the mAbs are not rheumatoid factors; that 36.2.2 is specific for α helical coiled-coil proteins such as myosin, actin, and keratin; and that 54.2.8 is an anti-DNA antibody.

Discussion

The presence of HRA in the sera of ARF patients has been a concern for several decades, and it has prompted investigators to identify streptococcal and host antigens that may participate in the disease process. For this reason, the most recent studies have focused on characterizing the antigens involved in the crossreactions between streptococci and heart tissue (14, 15, 18, 20). In our studies (14, 15, 18) and those of other investigators (4, 6) the cross reactive antigens of the streptococcus are found associated with membrane protein(s) and the major virulence determinant, M protein.

For this study, we chose M type 5 group A streptococci because of its frequent association with cases of rheumatic heart disease (24). The production and use of crossreactive murine mAbs as probes has been our strategy to resolve the identity of host and streptococcal antigens that immunologically crossreact. This approach led us to the identification of myosin as one of the autoantigens involved in these cross reactions. The stimulation of B cell clones against myosin by the streptococci is an attractive hypothesis for the production of cross reactive autoantibodies. This hypothesis is consistent with the work of Fischetti and Manjula (10, 11), which has shown that type 5 M protein resembles the structure of myosin, and the work of Dale and Beachey (25), which has shown that antimyosin antibody from ARF sera reacts with type 5 M protein.

Additional information about the specificity of our antistreptococcal mAbs has been presented herein and indicates that, in fact, the mAbs crossreactive with streptococci and myosin do react with a 54,000–56,000 mol wt piece of Pep M 5 protein, perhaps a dimer. The only other possible explanation of the larger-sized fragment is that it could be a protein sharing epitopes with M protein. This evidence does not preclude the reactivity of these antibodies with a 62,000–67,000 mol wt protein in the type 5 membrane preparations. Whether or not the membrane protein is part of the M protein molecule buried in the membrane is not yet clear. It is evident that our mAb reacted with both. These results may help to explain why there have been coincident and conflicting reports about the heart crossreactive antigens in both the cell wall and membrane preparations of group A streptococci (3, 5). Since small pieces of M protein were not strongly reactive with the mAbs, the M protein epitope may be associated with a conformational determinant, the dimeric form, or both. Alternatively, the epitopes reactive with our mAbs may be primarily confined to the carboxyl terminus of M protein and seen only on the largest piece of the pep M protein, since pepsin

extraction primarily cleaves the amino terminal half of the molecule, leaving behind the carboxy terminal residues. Conserved epitopes have been shown (26) to reside in the carboxyl terminus of M protein. This might explain the case for mAb 36.2.2 and 54.2.8, which reacted with both the pep M 5 and the whole M6 molecule, whereas 49.8.9 reacted only with pep M 5 in the ELISA. Further studies are in progress to elucidate the nature of these epitopes.

The most intriguing findings in this report center around the autoantigens, in addition to myosin, that react with these antistreptococcal mAbs. The most recent work by Fischetti and Manjula (10, 11) on the structure of streptococcal M proteins has identified them as α helical coiled-coil structures with a seven amino acid residue periodicity similar to that of tropomyosin, myosin, and the desmin-keratin family of α helical proteins. The results obtained with the mAb probes used in this study suggest that the crossreactive autoantigens are a family of α helical proteins or DNA.

There are very few studies of the presence of anti-DNA antibodies in ARF, but one study reported (28) that 29% of the ARF patients tested were antinuclear antibody (ANA)-positive. Another study of anti-DNA murine mAbs produced from mice susceptible to systemic lupus erythematosus reacted with endogenous bacteria, including *Streptococcus faecalis* (29). Some of these antibodies also reacted with the nucleus and with poly(I), poly(dT), and cardiolipin (30, 31). The antibodies found in mice susceptible to systemic lupus erythematosus appeared to be similar in activity to 54.2.8, and it could be speculated that they come from the same types of B cell clones. One report (31) has recently described an mAb to myosin that reacted with DNA, but whether or not it was an antibacterial antibody was not investigated. Since anti-DNA or antinuclear antibodies have been reported in autoimmune disorders (32), the amplification of antibody-producing clones directed at self antigens by infectious agents or microorganisms is a possible mechanism for the production of some of the autoantibodies (29, 33).

In conclusion, our studies provide definitive evidence that antistreptococcal mAbs react with streptococcal type 5 M protein and an unidentified streptococcal membrane protein(s), and with multiple autoantigens including DNA, myosin, keratin, and other α helical coiled-coil proteins. It is clear that the mAbs 36.2.2 and 54.2.8 have different specificities and that further investigation of crossreactive epitopes of the streptococcus and peptides of the α helical coiled-coil proteins such as myosin and M protein will be important in understanding the role of the streptococcus in the production of autoantibodies in rheumatic heart disease.

Summary

mAbs produced by immunization of BALB/c mice with *Streptococcus pyogenes* M type 5 membranes were further characterized for their reaction with *S. pyogenes* pep M5 protein and with autoantigens associated with human cell lines. mAbs 36.2.2 and 54.2.8 simultaneously reacted with M protein and a membrane protein(s) of *S. pyogenes*. When cell lines were mixed with 54.2.8, we saw nuclear fluorescence along with staining of the cytoskeleton. Subsequent experiments revealed that 54.2.8 was an anti-DNA antibody that reacted with DNA, poly(I), poly(dT), and weakly with cardiolipin. Its reactivity with the cytoskeleton could

be blocked with anti-vimentin. On the other hand, 36.2.2 reacted with the cytoskeleton, sparing the nucleus, and was inhibited by the α helical proteins myosin, actin, and keratin. mAb 54.2.8 was inhibited with myosin, but not with actin and keratin. None of the antibodies studied were inhibited by collagen, and none of them were rheumatoid factors. The results imply that Group A streptococci can activate B cell clones against myosin, α helical proteins, or DNA, thereby contributing to the enhancement of autoantibody production.

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References

1. Zabriskie, J. B., K. C. Hsu, and B. C. Seegal. 1970. Heart reactive antibody associated with rheumatic fever: characterization and diagnostic significance. *Clin. Exp. Immunol.* 7:147.
2. Kaplan, M. H., R. Bolande, L. Rakita, and J. Blair. 1964. Presence of bound immunoglobulins and complement in the myocardium in acute rheumatic fever. *N. Engl. J. Med.* 271:637.
3. Zabriskie, J. B., and E. H. Freimer. 1966. An immunological relationship between the group A streptococcus and mammalian muscle. *J. Exp. Med.* 124:661.
4. 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.
5. Kaplan, M. H. 1963. Immunologic relation of streptococcal and tissue antigens. I. Properties of an antigen in certain strains of group A streptococci exhibiting immunologic cross-reaction with human heart tissue. *J. Immunol.* 90:595.
6. Dale, J. B., and E. H. Beachey. 1982. Protective antigenic determinant of streptococcal M protein shared with sarcolemmal membrane protein of human heart. *J. Exp. Med.* 156:1165.
7. Lyampert, I. M., N. A. Borodiyuk, and G. A. Ugryumova. 1968. The reaction of heart and other organ extracts with the sera of animals immunized with group A streptococci. *Immunology.* 15:845.
8. Lyampert, I. M., L. V. Beletskrya, N. A. Borodiyuk, E. V. Gnezditskaya, I. I. Rassokhina, and T. A. Danilova. 1976. *Immunology.* 31:47.
9. Poirer, T. P., M. A. Kehoe, J. B. Dale, K. N. Timmis, and E. H. Beachey. 1985. Expression of protective and cardiac tissue cross-reactive epitopes of type 5 streptococcal M protein in *Escherichia coli. Infect. Immun.* 48:198.
10. Fischetti, V. A., and B. N. Manjula. 1982. Biologic and immunologic implications of the structural relationship between streptococcal M protein and Mammalian tropomyosin. *Semin. Infect. Dis.* 4:411.
11. Manjula, B. N., B. L. Trus, and V. A. Fischetti. 1985. Presence of two distinct regions in the coiled-coil structure of the streptococcal pep M5 protein: relationship to mammalian coiled-coil proteins and implications to its biological properties. *Proc. Natl. Acad. Sci. USA.* 82:1064.
12. Husby, G., I. van de Rijn, J. B. Zabriskie, Z. H. Abdin, and R. C. Williams, Jr. 1976.

Antibodies reacting with cytoplasm of subthalamic and caudate nuclei neurons in chorea and acute rheumatic fever. *J. Exp. Med.* 144:1094.

13. Kaplan, M. H. 1980. Streptococcal cross reactions with tissue antigens: cardiac myofibers, smooth muscle, and heart valve fibroblasts. Significance for pathogenesis in rheumatic fever. *In* Streptococcal Diseases and the Immune Response. S. E. Read and J. B. Zabriskie, editors. Academic Press, New York. 625.
14. Krisher, K., and M. W. Cunningham. 1985. Myosin: a link between streptococci and heart. *Science (Wash. DC).* 227:413.
15. Cunningham, M. W., N. K. Hall, K. K. Krisher, and A. M. Spanier. 1986. A study of anti-group A streptococcal monoclonal antibodies cross reactive with myosin. *J. Immunol.* 136:293.
16. van de Rijn, I., and R. E. Kessler. 1980. Growth characteristics of group A streptococci in a new chemically defined medium. *Infect. Immun.* 27:444.
17. van de Rijn, I., A. S. Bleiweiss, and J. B. Zabriskie. 1976. Antigens in *Streptococcus mutans* cross reactive with human heart muscle. *J. Dent. Res.* 55:59.
18. Cunningham, M. W., K. Krisher, and D. C. Graves. 1984. Murine monoclonal antibodies reactive with human heart and group A streptococcal membrane antigens. *Infect. Immun.* 46:34.
19. Manjula, B. N., and V. A. Fischetti. 1980. Studies on group A streptococcal M proteins: purification of type 5 M-protein and comparison of its amino terminal sequence with two immunologically unrelated M proteins molecules. *J. Immunol.* 124:261.
20. Cunningham, M. W., and S. M. Russell. 1983. Study of heart reactive antibody in antisera and hybridoma culture fluids against group A streptococci. *Infect. Immun.* 42:531.
21. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets; procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350.
22. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.
23. Naehlas, M. M., B. Monis, D. Rosenblatt, A. M. Sehgman. 1960. Improvement in the histochemical localization of leucine aminopeptidase with a new substrate, L-leucine-4-methoxy-2-naphthylamide. *J. Biophys. Biochem. Cytol.* 7:261.
24. Stollerman, G. H. 1982. Global changes in Group A streptococcal diseases and strategies for their prevention. *Adv. Intern. Med.* 15:373.
25. Dale, J. B., and E. H. Beachey. 1985. Epitopes of streptococcal M proteins shared with cardiac myosin. *J. Exp. Med.* 162:583.
26. Jones, K. F., B. N. Manjula, K. H. Johnston, S. K. Hollingshead, J. R. Scott, and V. A. Fischetti. 1985. Location of variable and conserved epitopes among the multiple serotypes of streptococcal M protein. *J. Exp. Med.* 161:623.
27. Leiber, M., B. Smith, A. Szakal, W. Nelson-Rees, and G. J. Tadara. 1976. A continuous tumor cell line from human lung carcinoma with properties of type II alveolar epithelial cells. *Int. J. Cancer.* 17:62.
28. Das, S. K., J. T. Cassidy, and R. E. Petlz. 1972. Antibodies against heart muscle and nuclear constituents in cardiomyopathy. *Am. Heart J.* 83:159.
29. Carroll, P., Stafford, R. S. Schwartz, and B. D. Stollar. 1985. Murine monoclonal anti-DNA antibodies bind to endogenous bacteria. *J. Immunol.* 135:1086.
30. Adrezejewski, C., Jr., J. Rauch, E. Lafer, B. D. Stollar, and R. S. Schwartz. 1980. Antigen binding diversity and idiotypic cross-reactions among hybridoma autoantibodies to DNA. *J. Immunol.* 126:226.
31. Lafer, E. M., J. Rauch, C. Andrzejewski, Jr., D. Mudd, B. Furie, R. S. Schwartz, and

B. D. Stollar. 1981. Polyspecific monoclonal lupus autoantibodies reactive with both polynucleotides and phospholipids. *J. Exp. Med.* 153:897.

32. Dighiero, G., P. Lymberi, J. C. Mazie, S. Rouyre, G. S. Butler-Browne, R. G. Whalen, and S. Avrameas. 1983. Murine hybridomas secreting natural monoclonal antibodies reacting with self antigens. *J. Immunol.* 131:2267.
33. Shoenfeld, Y., and R. S. Schwartz. 1984. Immunologic and genetic factors in autoimmune diseases. *N. Engl. J. Med.* 311:1019.