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Study of Heart-Reactive Antibody in Antisera and Hybridoma Culture Fluids Against Group A Streptococci

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Heart-reactive antibody is known to be produced in animals immunized with group A streptococci. However, the detection and quantitation of these antibodies and their respective antigens have been limited to immunofluorescence or immunoprecipitation tests. In this study a more sensitive technique was used to detect heart-reactive antibody, i.e., the enzyme-linked immunosorbent assay (ELISA). Countercurrent immunoelectrophoresis and agar gel immunodiffusion were also used to confirm the reactivity of rabbit antisera to group A streptococci and human heart tissue. Human heart tissue antigen was prepared by Triton X-100 extraction, and checkerboard titrations of heart tissue antigen versus streptococcal antisera revealed optimal concentrations of each for the ELISA. When immune rabbit serum was reacted with heart tissue antigen, a 5- to 10-fold increase was observed over the reactions of preimmune sera controls. Streptococcal antiserum was found to have a six- to eightfold greater reactivity with heart tissue antigen than with similar concentrations of kidney and skeletal muscle antigens. Heart tissue antigen was partially purified by DEAE-cellulose chromatography, and quantities of >10 to 100 ng (dry weight) were shown to react with streptococcal antisera in the ELISA. Heart, kidney, and skeletal muscle antigens were subjected to electrophoresis in polyacrylamide gel slabs and blotted onto a nitrocellulose filter. These filter blots reacted with streptococcal antisera and confirmed the tissue antigen specificity observed with the ELISA. In addition, the ELISA was found to be an effective method for the detection of heart-reactive antibodies produced by murine hybridomas that were producing antibody to group A streptococci.

Heart-reactive antibody is present in the sera of patients after acute rheumatic fever, poststreptococcal acute glomerulonephritis, and uncomplicated streptococcal infection (9, 23). It is well known that immunization of rabbits with *Streptococcus pyogenes* induces the production of antibodies that bind to heart tissue (10, 22). The detection of heart-reactive antibodies has usually been accomplished by the indirect immunofluorescence test or by immunoprecipitation tests (8; 14); however, both of these tests are limited by the quantity of antibody and antigen required for visible reactivity. The precipitation test is even more limited because not all streptococcal antisera produce visible precipitates when reacted with heart tissue antigen (HTA) (3). Therefore, we have sought a method which is sensitive, relatively simple, and capable of accommodating large numbers of antibody or antigen preparations.

In the present study, we have used the enzyme-linked immunosorbent assay (ELISA) technique to detect heart-reactive antibodies in rabbit antisera against *S. pyogenes* and to quantitate HTA. Western immunoblots (16) were used to confirm the reaction between streptococcal antisera and human tissues. In addition, the ELISA facilitated the detection of heart-reactive antibodies in murine hybridoma culture fluids that were also reactive with group A streptococci.

MATERIALS AND METHODS

Streptococci. The strains of *S. pyogenes* used in this study included M type 5 (Manfraedo), obtained from Edwin Beachey, Veterans Administration Hospital, Memphis, Tenn., and M type 25 ($T25_3$), obtained from Joseph J. Ferretti, University of Oklahoma Health Sciences Center, Oklahoma City, Okla. Stock cultures were frozen (−70°C) in Todd-Hewitt broth.

Antisera. Streptococcal antiserum to M type 5 was produced in rabbits by immunization with whole, UV-killed streptococci by a method previously described (4). Antiserum to M type 25 was a gift from Joseph J. Ferretti and was produced in rabbits by a similar method. Streptococci were grown in Todd-Hewitt broth or Todd-Hewitt broth dialysate;, after centrifu-

gation the cells were washed three times in phosphate-buffered saline (PBS) and were suspended in sterile PBS and used as immunogen.

Tissue antigen preparations. Normal tissues were obtained from surgery or autopsy and frozen at −70°C. The tissue was thawed and then homogenized in phosphate buffer (pH 7.0) containing 4% Triton X-100 (0.2 g [wet weight] per ml). Homogenization was performed for 3 min at 0°C in a Sorvall Omnimixer. The homogenate was then centrifuged at 12,000 × *g* in a Sorvall RC-2B centrifuge, and the supernatant was frozen in 1- to 2-ml samples at −70°C. Tissues were routinely tested for bacterial contamination, and only sterile samples were used. Protein determinations of tissue antigen preparations were performed by the Bio-Rad assay and reagent as previously described (2).

Agar gel immunodiffusion and CIE. Tissue antigen and streptococcal antibodies were reacted in immunodiffusion and countercurrent immunoelectrophoresis (CIE) tests. Immunodiffusion was performed in agar gel containing 0.8% purified agarose and 0.3% Noble agar in glycine-saline buffer (0.1 M glycine, 0.15 M NaCl), pH 8.2. Wells were cut in the agar gel plates and filled with appropriate antigen and antibody. These plates were incubated at 37°C for 24 to 48 h and washed in 0.1 M NaCl for at least 48 h with a final rinse in distilled water.

CIE was performed by the method of Anhalt et al. (1). Agar gel plates, which were prepared on Gel Bond (Marine Colloids, Inc., Rockland, Maine), contained 1% agarose in 0.05 M barbital buffer (pH 8.6). Opposing 3-mm wells were cut in the gel, and antisera and antigen were added to the wells. The antigen migrated toward the positive electrode during electrophoresis in 0.05 M barbital buffer (pH 8.6). After electrophoresis, the gels were soaked in 0.1 M NaCl for at least 48 h with a final rinse in distilled water. Gels were air dried overnight and subsequently stained for 15 min with Coomassie brilliant blue (1 g in 90 ml of ethanol, 20 ml of acetic acid, and 90 ml of water). Dried gels were destained in the ethanol-acetic acid-water mixture for 30 min and then air dried, and precipitin bands were recorded.

ELISA. The ELISA method used was that of Voller et al. (20). Tissue antigens were diluted in a carbonate-bicarbonate buffer (pH 9.6) and were coated overnight onto polyvinyl chloride microplates (Dynatech Laboratories, Inc.). Antibody dilutions were prepared in PBS–Tween 20 buffer and were reacted for 2 h with the antigen in the microplates. Goat anti-rabbit immunoglobulin G (IgG) alkaline phosphatase conjugate (Sigma Chemical Co.) was then reacted in the plates for 2 h. Plates were washed three times between each step with PBS–Tween 20 buffer. A positive reaction was indicated by the release of paranitrophenol and the development of a yellow color after the addition of the substrate paranitrophenyl phosphate. Optical density was measured at 405 nm in a Biotek ELISA reader.

Streptococci were bound to ELISA plates by glutaraldehyde fixation as described previously (20). Streptococci were grown in chemically defined medium (18) or Todd-Hewitt broth and washed three times in sterile PBS. Streptococci were suspended in PBS to a concentration of 0.25%, and 50 μl was pipetted into wells of polyvinyl chloride microplates. Plates were centrifuged for 1 min at 100 × *g* and fixed with 0.25% glutaraldehyde for 5 to 7 min. The glutaraldehyde solution was decanted, and plates were washed three times with PBS. Subsequently, 3% bovine serum albumin (BSA) was added to each well and plates were sealed and stored at 4°C. The ELISA performed in plates containing bound streptococci was by the procedure described above, except that the wash and dilution buffer was PBS without Tween 20.

Murine hybridoma production. BALB c/BYJ mice (Jackson Laboratories) were immunized intraperitoneally with either UV-killed, whole M type 5 *S. pyogenes* cells or 200 μg of purified M type 5 membranes. *S. pyogenes* for immunization was grown in chemically defined medium as previously described (18). Membranes were prepared as described previously (17), and both whole cells and membranes were emulsified 1:1 in Freund incomplete adjuvant. Mice were immunized intraperitoneally and given booster injections three times after the initial injection. At 2 to 3 days after the last booster dose, spleens were removed aseptically from the mice and minced through sterilized 100-mesh screen. The mincings were washed in Iscove modified Dulbecco medium with 20% horse serum and 1% gentamicin sulfate. Spleen cells were centrifuged at 400 × *g* for 7 min, and the supernatant was decanted. The cells were suspended in Iscove modified Dulbecco medium with 20% horse serum (10 ml per spleen), and cell counts were performed in trypan blue to determine viable cell numbers.

Optimal conditions established by Goding for producing hybridomas were used in these experiments (7). Murine myeloma cells (cell line × 63-Ag 8.6.5.4, a variant from the original P3 × 53-Ag-8 cell line) were mixed with approximately equal numbers of spleen cells. After mixing, the cells were centrifuged at 400 × *g* for 7 min. The supernatant was decanted, and 3 ml of 35% sterile polyethylene glycol (pH 7.8) was added. The pellet was gently shaken and left undisturbed for 6 min at 30°C, after which it was centrifuged, suspended in Iscove modified Dulbecco medium plus 20% horse serum, and incubated overnight at 37°C and 8% CO_2. One day after fusion, cells were plated into conditioned, 96-well sterile microtiter plates (Costar Laboratories). Iscove modified Dulbecco medium containing 20% horse serum, 1×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine was used to feed the cells in the plates every 2 days for 2 weeks. When culture fluids became acidic, they were tested in the ELISA as described above with either M type 5 *S. pyogenes* or HTA as the antigen.

Polyacrylamide gel electrophoresis and immunoblot techniques. Slab gel electrophoresis was performed essentially as described by Laemmli (11) and Studier (15). Samples were prepared for electrophoresis by boiling for 2 min in a solution containing 0.05 M Tris-hydrochloride, 1% sodium dodecyl sulfate, 1% mercaptoethanol, 0.002 M EDTA, 10% glycerol, and bromphenol blue. Molecular weight standards were included in each experiment.

Samples were applied onto a 5% polyacrylamide spacer gel (pH 6.8) for electrophoresis into polyacrylamide slab gel gradients (10 to 20% acrylamide) containing 0.375 M Tris-glycine buffer (pH 8.8) with 0.1% sodium dodecyl sulfate and 0.002 M EDTA. Tris-glycine buffer (0.05 M Tris, 0.192 M glycine), pH 8.3, containing 0.1% sodium dodecyl sulfate and 0.002 M EDTA was used for the electrode buffer. Constant current was maintained at 35 mA for 8 to 9 h. When the

indicator bromphenol blue reached the bottom of the gel, the gel was removed, and a side portion (five to six lanes) was washed overnight in 50% methanol. Gels were stained in silver nitrate by the method of Wray et al. (21). The other portion of the gel was blotted (Bio-Rad Trans-Blot apparatus) overnight onto a nitrocellulose membane (0.45-μm pore size) with a constant current maintained at 75 mA. The Tris-glycine blotting transfer buffer contained 20% methanol, 0.192 M glycine, 0.025 M Tris (pH 8.3), and 0.01% sodium dodecyl sulfate. The blotting procedure was as described by Towbin et al. (16). When electrophoretic transfer of proteins was complete, a side portion of the blot was stained with 0.2 g of amido black in a mixture of 90 ml of methanol, 20 ml of acetic acid, and 90 ml of distilled water. After staining for 3 min, the blot was destained in a mixture of 450 ml of methanol, 20 ml of acetic acid, and 90 ml of distilled water. The other portion of the blot was reacted with 3% BSA in PBS for 1 h at 37°C. The BSA-saturated sheets were reacted with a 1:10 dilution of rabbit antiserum for 1 to 2 h. Dilutions of sera were prepared in 3% BSA. After incubation with antisera, the blots were washed five times with 100-ml amounts of PBS for 30 min to 1 h. The nitrocellulose membranes were then reacted with peroxidase-labeled goat anti-rabbit IgG in 3% BSA for 1 h and washed five times with PBS. Finally, peroxidase-labeled rabbit antiperoxidase was reacted with the nitrocellulose membrane for 40 min and washed as above. This procedure is similar to that of Glass et al. (6). Antigenic bands were observed after the addition of the substrate 4-chloro, 1-napthol by the method of R. C. Briggs (personal communication). The substrate was prepared by dissolving 25 mg in 0.5 ml of 95% ethanol. The substrate was then mixed with 100 ml of 0.05 M Tris buffer (pH 7.4) and stirred in the dark for 30 min. After stirring, the substrate was filtered and used immediately after addition of 17 μl of 30% H_2O_2. Antigenic bands stained a deep purple color in 3 to 5 min. Blots were rinsed in distilled water and dried.

RESULTS

Reactivity of HTA with streptococcal antisera in immunodiffusion and CIE. In preliminary experiments the reaction of group A streptococcal anti-M type 5 and anti-M type 25 sera with HTA was positive in the CIE. Five HTAs were used in the CIE against both anti-M type 5 and anti-M type 25 sera. Both antisera produced positive precipitin reactions in the CIE with the five HTAs tested. Anti-M type 5 reactions in the CIE were not as strong as those seen with anti-M type 25 serum, and occasionally anti-M type 5 serum was unreactive. Normal rabbit serum did not react with HTA. In immunodiffusion tests, anti-M type 25 serum produced one precipitin band when reacted with HTA, however, anti-M type 5 serum was unreactive. Anti-M type 25 serum produced more positive reactions in precipitation tests with HTA than did anti-M type 5 serum.

Partial purification of HTA. For use in the ELISA, the crude HTA was partially purified by chromatography of the tissue extract on diethylaminoethyl cellulose (pH 8.0). The column was washed with 0.001 M sodium phosphate buffer (pH 8.0), and peaks 1 and 2 were eluted. When an NaCl gradient from 0.001 to 0.5 M was applied to the column, peaks 3 and 4 were eluted. Fractions were measured for absorbance at 280 nm, and the four peaks were individually pooled, dialyzed against distilled water, and lyophilized. Both pools 1 and 2 produced one precipitin band when electrophoresed in CIE against anti-M type 25 serum indicating that serologically reactive antigen was released from the column in the 0.001 M phosphate buffer wash (Fig. 1). When reacted with anti-M type 25 serum in immunodiffusion, pool 1 produced a line of identity when compared with crude HTA. Pools 2, 3, and 4 were not reactive in the immunodiffusion test. Therefore, the HTA in pool 2 was apparently in much lower concentration than in pool 1. The material recovered in pool 1 was used to quantitate the amount of HTA reactive in ELISA tests. Pools 2, 3, and 4 yielded only small quantities of lyophilized material and were not tested in the ELISA.

Titration of HTA and anti-M type 5 *S. pyogenes* serum with the ELISA. Optimal concentrations of HTA and anti-M type 5 serum used in the ELISA were determined by checkerboard titration. Dilutions of HTA were reacted with anti-M type 5 serum diluted 1:10, 1:50, 1:100, and 1:200. The optimal concentration of antiserum was found at a 1:10 dilution and was used at this concentration throughout the study. The values determined by the ELISA for both immune anti-M type 5 and preimmune sera are presented in Table 1. Preimmune serum produced low-level reactions with HTA, and in two different rabbits, immune serum reactivity with HTA increased 5- to 10-fold over that of preimmune serum controls. The greatest difference between immune and preimmune sera occurred between HTA dilutions 1:250 and 1:1,024. Therefore, optimal HTA activity was found between a 1:250 and 1:1,024 dilution. These dilutions were compatable to approximately 240 and 60 μg of protein per well, respectively. The omission of Tween-20 in the PBS wash and dilution buffer resulted in decreased reactivity of heart, skeletal muscle, and kidney with streptococcal antibody. For this reason Tween-20 was routinely used in the PBS wash and dilution buffer.

Specificity of the reaction between HTA and antisera against *S. pyogenes* types 5 and 25. Anti-M type 5 and anti-M type 25 sera were examined for their reactivity with kidney and skeletal muscle. Antiserum diluted 1:10 was reacted in the ELISA with HTA at protein concentrations of 600, 300, 120, and 60 μg per well. These protein concentrations are comparable to dilutions of 1:100, 1:200, 1:500, and 1:1,000, respec-

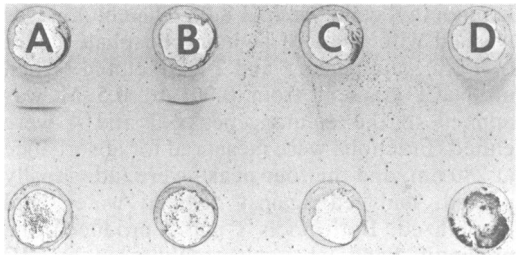

FIG. 1. CIE of anti-M type 25 *S. pyogenes* serum in bottom wells versus diethylaminoethyl pools 1 through 4 in wells A through D.

tively (Fig. 2). HTA demonstrated six- to eight-fold greater reactivity than the same weight and protein concentrations of kidney and skeletal muscle antigens at 600 and 300 μg per well. The values in Fig. 2 represent the difference between immune and preimmune serum reactions. Similar results were observed with anti-M type 5 serum. The most significant difference between the reactivity of streptococcal antisera with heart, skeletal muscle, and kidney preparations was observed at 120 to 600 μg of protein per well. At 60 μg/well (1:1,000 dilution) skeletal muscle and kidney became reactive with immune sera, but remained unreactive with preimmune serum controls. Tissue antigens were then diluted to 10^{-10} and reacted with anti-M type 25 sera to determine the full range of activity with HTA and kidney and skeletal muscle antigens (Fig. 3). The HTA and skeletal muscle activity peaked at a dilution of 10^{-3}, but the kidney antigen optimum was at a 10^{-4} dilution. HTA was reactive over a wider range of dilutions than were kidney and skeletal muscle antigens. Effective detection occurred at protein concentrations as low as 600 ng (10^{-5} dilution). Skeletal muscle and kidney preparations were less reactive than HTA, but showed similar optimum concentrations. These results demonstrate the importance of the antigen concentration for optimal reactivity in the ELISA. Similar results were observed with a different human heart preparation and with 3% BSA incorporated into the ELISA buffer washes and serum dilutions.

Partially purified HTA in concentrations from 10 to 100 ng to 100 μg (dry weight) were reacted with anti-M type 5 serum. The plot of optical density versus concentration of antigen was linear to 100 μg (Fig. 4). Therefore, the assay proved to be extremely sensitive even though the HTA was only partially purified.

Utilization of the ELISA to test murine hybridomas for reactivity with group A streptococci and heart. Hybridomas prepared from spleens of mice immunized with group A streptococci or their membranes were tested for antibody production against M type 5 *S. pyogenes* and HTA. These hybridomas produced antibodies in culture fluid which reacted with HTA or type 5 streptococci (or both) in the ELISA (Table 2). Two types of hybridoma antibodies were identified as those directed against streptococcal antigens only and those directed against both streptococci and HTA. The ELISA was effective at screening these hybridomas for activity.

Reactivity of S. pyogenes anti-M type 5 and anti-M type 25 sera with western blots of electrophoresed tissue antigens. To distinguish differences in specificities of anti-M type 5 and anti-M type 25 heart-reactive antibodies, HTA and kidney and skeletal muscle antigens were applied to polyacrylamide slab gels for electrophoresis, and immunoblots were performed with streptococcal antisera. A portion of the slab gel was stained with silver nitrate, and proteins of a wide molecular weight range were observed in HTA preparations. The other portion of the gel was

TABLE 1. Alkaline phosphatase ELISA of crude HTA versus anti-M type 5 *S. pyogenes* serum[a]

Dilution of crude HTA	Optical density at 400 nm			
	Anti-M type 5 serum I	Preimmune serum I	Anti-M type 5 serum II	Preimmune serum II
1:32	0.608	0.114	0.449	0.133
1:64	0.803	0.04	0.518	0.138
1:125	0.751	0.069	0.404	0.168
1:256	1.041	0.038	0.767	0.215
1:512	1.005	0.046	0.815	0.174
1:1,024	0.894	0.017	0.390	0.178
1:2,048	ND[b]	0.06	0.427	0.213
1:4,096	0.403	0.03	0.228	0.153
1:8,192	0.438	0.02	0.144	0.148
1:16,384	0.442	0.008	0.246	0.111
Antigen 1:32 + PBS	0.079			
Antibody 1:10 + PBS	0.016	0.035	0.090	0.029

[a] Antiserum was diluted 1:10.
[b] ND, Not done.

blotted onto nitrocellulose sheets. A portion of the blot, containing heart, kidney, and skeletal muscle protein bands was stained with amido black, and other portions containing these same proteins were reacted separately with normal rabbit serum, anti-M type 5 and anti-M type 25 *S. pyogenes* sera. Peroxidase-labeled antibody conjugates and substrate were reacted with the blot. The amido black stained the blotted proteins of heart, kidney, and skeletal muscle (Fig. 5). Many faintly reacting bands were observed in immunoblots of NRS and immune sera, however, the strongly reacting bands of the immune sera were obvious when compared with normal rabbit serum. A protein at approximately 40,000 daltons in heart and skeletal muscle, but absent from kidney, produced the most striking reaction with anti-M type 5 serum. It reacted faintly with normal rabbit serum and anti-M type 25 serum. In addition, a high-molecular-weight protein only in the heart reacted with anti-M type 5 serum. On the other hand, anti-M type 25 serum produced the strongest reaction with two heart, kidney, and skeletal muscle proteins found above 40,000 and 67,000 daltons, respectively. Anti-M type 5 serum also reacted with a protein above 67,000 daltons. Another molecule, at approximately 30,000 daltons and present in high concentrations in skeletal muscle, reacted strongly with normal rabbit serum as well as with anti-M type 5 and anti-M type 25 sera. These reactions confirmed the reactivity of streptococcal antisera with heart, kidney, and skeletal muscle in the ELISA and demonstrate the presence of antibodies in streptococcal antiserum which appear to react with more than one HTA.

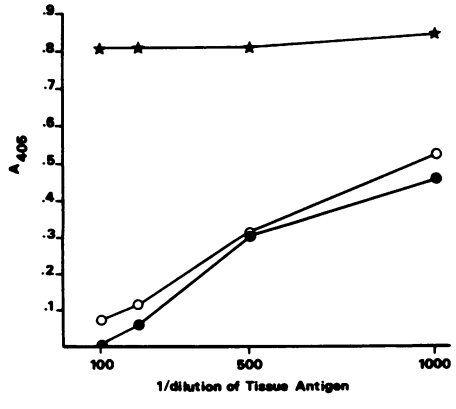

FIG. 2. ELISA determination of tissue reactive antibody in anti-M type 25 *S. pyogenes* serum. Symbols: (★) heart, (○) skeletal muscle, (●) kidney. A_{405}, Absorbancy at 405 nm.

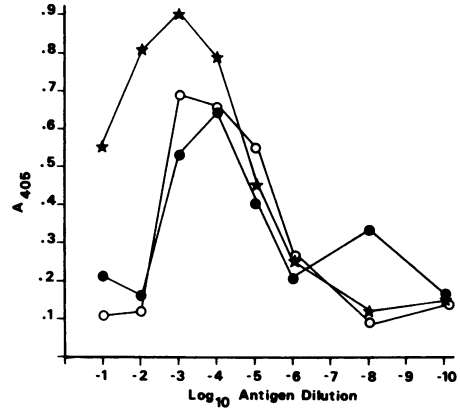

FIG. 3. ELISA determination of tissue reactive antibody in anti-M type 25 *S. pyogenes* versus (★) heart, (○) skeletal muscle, and (●) kidney diluted 10^{-1} to 10^{-10}. A_{405}, Absorbancy at 405 nm.

DISCUSSION

Numerous studies have demonstrated heart-reactive antibodies in group A streptococcal antisera (8, 14, 22). Either indirect immunofluorescence (8, 22) or imunoprecipitation techniques (8, 14) were used to observe these reactions. In our study, precipitation reactions observed between heart tissue and rabbit anti-streptococcal sera were positive, but were not consistently present in immunodiffusion tests for all of the sera studied. CIE was required to obtain positive precipitin reactions in the case of the anti-M type 5 serum. There have been no reports of a sensitive and rapid method for the detection and study of streptococcal antiserum and its reaction with tissue antigens. Our study demonstrates that an ELISA was an effective detector of heart-reactive antibodies present in rabbit antisera against *S. pyogenes*. The streptococcal antibodies were specific for heart antigens only in the higher range of antigen concentrations tested. Reactivity of antibodies in streptococcal antisera with kidney and skeletal muscle antigens was also observed, but at lower concentration ranges of antigen and to a lesser degree than with heart. These results most likely reflect different antibody specificities or affinities.

In ELISA tests, optimal antigen concentrations were crucial to obtain maximum reactivity. Skeletal muscle was more reactive than kidney with anti-M type 5 streptococcal serum. Similar results were observed with culture fluids from murine hybridoma polyclones when reacted with kidney and skeletal muscle. The ELISA was extremely sensitive, which explains the ease with which other antigens in kidney and skeletal muscle were detected when at optimum concentrations. Moreover, the ELISA easily

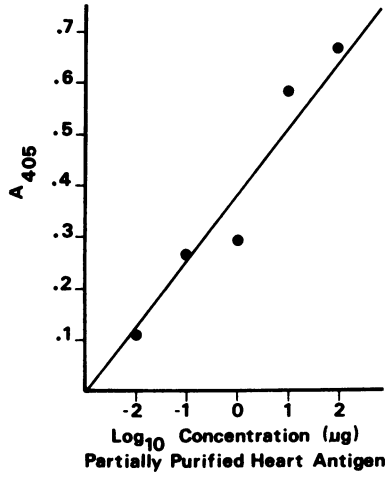

FIG. 4. ELISA determination of heart-reactive antibody in anti-M type 5 *S. pyogenes* serum versus increasing concentrations of partially purified HTA diethylaminoethyl pool I. A_{405}, Absorbancy at 405 nm.

distinguished those hybridomas producing streptococcal and heart-reactive antibody.

It is not new knowledge that streptococcal antiserum reacts with other tissues or with a variety of animal hearts. Antiserum against group A streptococci has been reported to react with connective and renal tissue, brain, liver, and heart and skeletal muscle (12, 13). Sarcolemmal and subsarcolemmal antigens which react with streptococccal antisera have been observed in human, rabbit, guinea pig, rat, and mouse heart tissues (13). In our study, similar results were observed with different human hearts in the ELISA. In addition, incorporation of 3% BSA into ELISA buffer and test serum dilutions did not alter the results. Therefore, we propose that the reactions observed in the ELISA were specific and represent antigen(s) in heart, skeletal muscle, and kidney which react with antibodies in antistreptococcal rabbit serum.

Antiserum produced in rabbits immunized with Todd-Hewitt broth grown *S. pyogenes* was not investigated for reactivity with Todd-Hewitt broth. It is not known whether any of the heart-reactive antibody in rabbit antiserum was against Todd-Hewitt broth. However, *S. pyogenes* cells grown in chemically defined medium or purified *S. pyogenes* membranes were used as immunogens for hybridoma production. The culture fluids of these hybridomas produced results similar to those observed with rabbit antisera against Todd-Hewitt broth grown *S. pyogenes*. Therefore, we suggest that these tissue-reactive antibodies present in rabbit sera were induced by a streptococcal antigen.

Results from immunoblots of human HTA and kidney and skeletal muscle antigens show that various amounts of different reactive antigens exist in these tissues. In fact, one protein reactive with anti-M type 5 *S. pyogenes* serum was absent or in very low concentration in kidney, but present in heart and skeletal muscle. More strongly reactive antigen(s) were found in immunoblots of heart and skeletal muscle than in those of kidney when anti-M type 5 serum was reacted with the blots. Anti-M type 5 and anti-M type 25 *S. pyogenes* sera produced noticeably different reactions when their immunoblots of heart, kidney, and skeletal muscle were compared. This is not surprising since at least two cross-reactive antigens have been identified with group A streptococci. The type 5 M protein antigen was recently shown by Dale and Beachey (5) to induce the production of a type-specific opsonizing and heart-reactive antibody. In addition, a cross-reactive antigen has been partially purified from group A streptococcal membranes which appears to be associated with most group A organisms (19). In that recent study, heart-reactive antibody in rabbit streptococcal antiserum was not competitive with the heart reactivity of acute rheumatic fever serum. Whether this was due to two completely different antigens was not determined. However, the identification of the two antigen systems in group A streptococci lends credence to the fact that the two different *S. pyogenes* antisera used in this study reacted with different tissue antigens. The identification of differences in the strongest reactor proteins in the immunoblots and their significance in relation to reactions of

TABLE 2. Testing of murine hybridomas for immunological reactivity with M type 5 *S. pyogenes* and HTA in the ELISA

Hybridoma polyclone no.	Culture fluid reaction[a] with:	
	Streptococci	Heart
1	0.047	0.033
20	0.500	0.034
21	0.433	0.782
27	0.363	0.042
36	0.873	1.153
37	0.622	1.390
49	0.993	0.754
51	0.948	0.750
54	1.065	0.989
56	0.308	0.819
58	0.758	0.362
63	0.703	0.896
65	0.589	0.732

[a] Activity of polyclones in ELISA at 405 nm. The polyclones described are those that were transferred in cell culture several times and possessed stable activity.

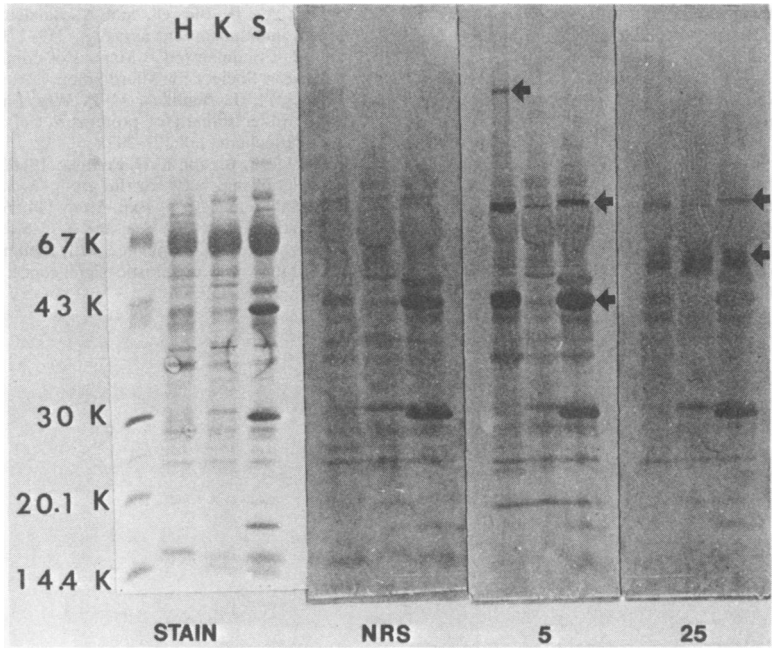

FIG. 5. Western immunoblots of electrophoresed HTA (H) and kidney (K) and skeletal muscle (S) antigens. A portion of the blot was stained with amido black (STAIN), and the other portions were reacted with normal rabbit serum (NRS), anti-M type 5 *S. pyogenes* serum (5), and anti-M type 25 *S. pyogenes* serum (25) and then with peroxidase-labeled conjugates. Molecular weight standards (far left lane of stain) from top to bottom are 67,000, 43,000, 30,000, 20,100, and 14,400.

acute rheumatic fever sera awaits further elucidation.

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