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Research Article

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Using the in vitro technique for the inhibition of capillary migration of peripheral blood leucocytes as an index of the degree of sensitivity to streptococcal antigens, the results indicate that patients with acute rheumatic fever exhibit an exaggerated cellular reactivity to these antigens and in particular to streptococcal cell membrane antigens. This abnormal response to streptococcal membrane antigens appears to persist in rheumatic subjects for at least 5 yr after the initial attack of rheumatic fever. Only Group A streptococcal membrane antigens elicited this unusual response in rheumatic subjects, since the cellular reactivity to Group C and D streptococcal membranes was the same in all groups. Patients with evidence of valvular disease exhibited the same degree of cellular reactivity to these antigens as did patients without clinical evidence of rheumatic heart disease.

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Cellular Reactivity Studies to Streptococcal Antigens

MIGRATION INHIBITION STUDIES IN PATIENTS WITH STREPTOCOCCAL INFECTIONS AND RHEUMATIC FEVER

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ABSTRACT The question of whether hypersensitivity to streptococcal antigens plays a role in the pathogenesis of the nonsuppurative sequelae of streptococcal infections remains at present unclear. As a first step in the approach to this question, the degree of cellular reactivity of peripheral blood leucocytes to streptococcal antigens was investigated in a number of rheumatic fever patients, patients with uncomplicated streptococcal infections, as well as normal healthy subjects.

Using the in vitro technique for the inhibition of capillary migration of peripheral blood leucocytes as an index of the degree of sensitivity to streptococcal antigens, the results indicate that patients with acute rheumatic fever exhibit an exaggerated cellular reactivity to these antigens and in particular to streptococcal cell membrane antigens. This abnormal response to streptococcal membrane antigens appears to persist in rheumatic subjects for at least 5 yr after the initial attack of rheumatic fever. Only Group A streptococcal membrane antigens elicited this unusual response in rheumatic subjects, since the cellular reactivity to Group C and D streptococcal membranes was the same in all groups. Patients with evidence of valvular disease exhibited the same degree of cellular reactivity to these antigens as did patients without clinical evidence of rheumatic heart disease.

The nature of the antigens responsible for the observed cellular response remains unknown. Enzymatic treatment of streptococcal cell walls and membranes designed to remove type-specific M proteins did not alter the observed cellular reactivity to the streptococcal antigens. The finding that an abnormal cellular response to certain streptococcal antigens is present only in rheumatic patients suggests that cell-mediated factors may play an important role in the disease process.

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INTRODUCTION

The question of whether hypersensitivity to hemolytic streptococci and their products might play a role in the pathogenesis of the nonsuppurative sequelae of streptococcal infections has been the subject of investigation for many years. Starting with the early work of Swift and Derick and Derick, Hitchcock, and Swift in animals (1–3), it was apparent that delayed hypersensitivity to streptococci behaved in a manner similar to that observed for tuberculin sensitivity (4). Initiation of sensitivity by prolonged focal contact between the intact bacteria and tissues of the host, inability to passively transfer streptococcal-delayed hypersensitivity with serum alone, and the lack of correlation between circulating antibodies to streptococcal products and delayed allergy were all characteristics of the streptococcal hypersensitive state (5). Apropos of these studies, several intriguing observations concerning the streptococcal hypersensitive state were made. First, repeated small inoculations of heat-killed streptococci were more effective in inducing the hypersensitive state than a single inoculation of living organisms (3). Secondly, extracts of streptococcal cellular structures were more effective in eliciting the hypersensitive reaction than streptococcal extracellular products. Thirdly, Möen clearly described specific cytotoxic and cellular inhibition effects by streptococcal antigens on explanted host cells from rheumatic patients in tissue culture, as opposed to cell cultures from normal controls. It is interesting that these observations were made at least 20 yr before the emergence of modern in vitro techniques of cellular immunology and cytotoxicity (6).

Experiments similar to those described in animals were also carried out in man by several investigators (7–9). In using skin tests as an index of delayed sensitivity to streptococcal products, the general consensus of the workers was that hypersensitivity to streptococci and their products was a common occurrence in man and increased in intensity, depending on the age of the individual tested. In general, these reactions were more intense in rheumatic subjects than in nonrheumatic controls (7, 8), the reactivity was particularly intense with extracts of hemolytic streptococci as compared to nonhemolytic streptococcal strains (8), and the greatest number of positive reactions were obtained with autogenous streptococci, suggesting some type specificity to the reaction (10). In this connection, Beachey, Alberti, and Stollerman (11) and Pachman and Fox (12) have recently observed that immunization of guinea pigs with partially purified preparations of different M proteins resulted in specific delayed hypersensitivity (skin tests and macrophage inhibition tests) to the immunizing antigen. However, cross-reactions to other type-specific M proteins were seen, although the reactions were always considerably less than those observed with the type-specific protein used for immunization.

Finally, the cellular nature of this streptococcal sensitivity reaction was clearly delineated by Lawrence (5) when he demonstrated that delayed hypersensitivity in man could be transferred to a streptococcal skin test-negative individual via extracts of peripheral blood white cells obtained from a streptococcal skin test-positive individual.

While these studies were primarily concerned with the kinetics of the induction of the delayed hypersensitivity state to streptococci and their products, other observations lent support to the concept that streptococcal hypersensitivity played a role in the nonsuppurative sequelae of streptococcal infections. In studies on experimental models of rheumatic fever in animals, a number of investigators (13, 14) have emphasized the need for repeated closely spaced streptococcal inoculations with a marked cutaneous reactivity to streptococcal materials as being a prime prerequisite in inducing the pathological lesions which simulated rheumatic lesions found in man. Rantz and others (15–18) have also suggested that, in man, repeated streptococcal infections are important for the disease process, and they cite the rarity of rheumatic fever before 3–4 yr of age as evidence for the necessity of an acquired hypersensitivity to streptococcal materials before rheumatic fever occurs.

Taken together, these studies strongly suggest that hypersensitivity to streptococcal antigens may play a role in the initiation of events leading to the disease rheumatic fever. The success in detecting delayed hypersensitivity to a variety of particulate antigens by using the technique of in vitro cellular migration of peripheral white blood cells (19, 20) prompted an investigation into the reactivity of rheumatic and nonrheumatic individuals to various streptococcal antigens. The following report indicates that patients with acute rheumatic fever are highly reactive to streptococcal cellular structures, in particular to the streptococcal membrane, and they maintain this hyper-reactive state for at least 5 yr after the initial attack.

METHODS

Patients. The majority of the rheumatic patients came from the Rheumatic Fever Service of The Rockefeller University Hospital. Some of the acute rheumatics were from other New York City hospitals. Normal controls, as well as patients with uncomplicated streptococcal infections, came from this same urban population and were matched for age and sex.

Streptococcal strains. All strains used in this study were obtained from The Rockefeller University collection and were kindly provided by Dr. Rebecca C. Lancefield.

Isolation of cellular structures. Lyophilized cultures of Group A streptococcal strains S43/197 (Type 6) and A964 (Type 5) were transferred to Todd-Hewitt broth containing 5% rabbit blood. After 18 h of incubation at 37°C, 0.5 ml of the supernate of the broth culture was transferred to 35 ml of dialysate medium prepared as previously described (21) and incubated another 18 h at 37°C. 10 ml of this culture was transferred to 2 liters of dialysate medium, and after 18 h of incubation, the contents of the 2-liter flask were inoculated into 20-liter batches of dialysate medium and incubated 18 h. Using high speed Sharples centrifugation of the 20-liter culture (Sharples-Stokes Div., Penwalt Corp., Warminster, Pa.), the cells were isolated from the broth and washed twice in isotonic saline, pH 6.0.

These cells were then divided into two portions. One portion was resuspended in distilled water, the cells disrupted in a Braun disintegrator (Bico, Inc., Burbank, Calif.), and the cell walls isolated by methods previously described (22). A small aliquot of this cell wall preparation was washed in distilled water, lyophilized, and the contents weighed to determine the dry weight of isolated cell walls in the preparation. Knowing the dry weight of the cell walls, the remaining cell wall pellet was diluted in 0.005 M phosphate-buffered saline, pH 7.5, to a concentration of 1 mg/ml and stored in 1-ml aliquots at −70°C until use. In this manner, thawing and refreezing of the cell wall preparation occurred only once, and only once-thawed preparations of cell walls were used for each experiment.

The remaining portion of streptococcal cells was resuspended in 0.05 M phosphate buffer, pH 6.1, containing 4% NaCl and 0.005 M EDTA with activated phage-associated lysin (13). The preparation of streptococcal protoplasts and isolation of the streptococcal membranes from this mixture was as previously described (21). The procedure for the storage of aliquots of these membranes was the same as that described above for the isolated streptococcal cell wall material. Isolation of cell walls and membranes of Group C streptococcal strain C74 was achieved by the methods described above.

Since the phage-associated lysin does not attack Group D streptococcal strains, cell walls and membranes of strains from this serogroup (Group D) were isolated by differential centrifugation after mechanical disruption of the streptococcal cells in a Braun disintegrator (22).

Enzymatic treatment of isolated streptococcal cellular structures. Trypsin experiments. Streptococcal cell walls or cell membranes at a concentration of 10 mg/ml were suspended in 10 ml of 0.1 M Tris buffer, pH 8.0, to which crystalline trypsin (Worthington Biochemicals Corp., Freehold, N. J.) was added in a final concentration of 100 μg/ml. This mixture was placed in Visking no. 24 dialysis tubing (Visking Corp., Chicago, Ill.) and the contents dialyzed against 500 ml of 0.1 M Tris buffer, pH 8.0, for 2½ h at 37°C with gentle agitation. The dialyzing buffer had been prewarmed to 37°C before insertion of the dialysis sac.

After this enzymatic digestion, the contents of the dialysis sac were centrifuged at 200 *g* for 20 min, washed twice in 0.05 M phosphate-buffered saline, pH 7.5, then washed twice in distilled water. An aliquot was removed, lyophilized, and weighed to determine the dry weight of the streptococcal cell wall and membrane preparations. The suspensions were recentrifuged and appropriately diluted to contain 1 mg/ml in each tube. All tubes were refrozen at −70°C until use.

Preparation of tissue culture medium. Stock solutions of tissue culture medium (Flow Laboratories, Inc., Rockville, Md.) were prepared as follows: under sterile conditions, 500 ml of Earle's balanced salt solution (catalogue no. 3012, 10× concn), 100 ml of minimal essential medium amino acids (catalogue no. 6-213, 50× concn), and 50 ml of minimal essential medium vitamins (catalogue no. 6-224, 100× concn) were mixed together, placed in sterile screw cap glass bottles, labeled "solution A", and kept at 4°C until use. For daily consumption, 130 ml of solution A was mixed with 30 ml of sterile 8% $NaHCO_3$, 100 ml of L-glutamine (catalogue no. 6-134, 100× concn), and sufficient sterile distilled water to make 1 liter. To this mixture, labeled "solution B", 100,000 U of penicillin, (Eli Lilly and Co., Indianapolis, Ind.), 100,000 μg of streptomycin (Eli Lilly and Co.), and 20,000 U of mycostatin (E. R. Squibb and Sons, Princeton, N. J.) were added. This solution was stored at 4°C until use. Aliquots of solution B were mixed with either 20% fetal calf serum obtained from Flow Laboratories (Flow Laboratories, Rockville, Md., catalogue no. 4055) or 10% normal AB human plasma. To prevent repeated freezing and thawing of stock serum solutions, these sera were prepared as follows: 500 ml of frozen fetal calf serum was thawed, heat inactivated for 30 min at 56°C, distributed in 20-ml aliquots, and refrozen at −20°C until use. Normal human AB plasma was separated from the blood cells by centrifugation, inactivated at 56°C for 30 min, and stored at −20°C in similar aliquots. On the day of use, aliquots of serum or plasma were removed, thawed, and mixed with medium to make the appropriate solutions. An additional 100 U/ml of penicillin were added to the complete medium just before use. While solution A was kept for long periods at 4°C, it was felt advisable not to store stock solutions of B more than 10 days at 4°C.

Isolation of peripheral white blood cells. 50 ml of venous blood was drawn from the median cephalic vein of the anticubital fossa of each patient into an evacuated rubber-stoppered bottle containing approximately 10–15 glass beads. The bottle was gently shaken for 10 min and the defibrinated blood was then poured into a graduated glass cylinder. An equal volume of a 2% Knox gelatin solution in Ringer's lactate was added to the volume of blood and the mixture allowed to sediment for 20 min at 37°C. The supernate, containing primarily leucocytes, was removed, placed in either plastic (Falcon Plastics, Los Angeles, Calif.) or glass tubes, and spun at 1,500 rpm for 10 min. The cell-tree supernate was removed and the cells from each tube pooled and resuspended in 10 ml of 0.83% ammonium chloride for approximately 5 min to lyse the contaminating red cells. The cells were again spun at 1,500 rpm, the fluid removed, and the cells washed twice in Ringer's lactate solution. After the final wash, the cells were resuspended in 10 ml of complete medium (see section on preparation of tissue culture medium). The cells were counted in a hemocytometer (Hausser and Son, Ace Scientific Supply Co., Inc., Linden, N. J.). The cell suspension was again spun at 1,500 rpm and the cell pellet adjusted to 20 million lymphocytes/ml. Nonheparinized capillary tubes (Arthur H. Thomas Co., Philadelphia, Pa.) were filled with aliquots from this cell suspension and sealed with Seal-Ease clay (Clay Adams, Div., Becton, Dickinson and Co., Parsippany, N. J.). The capillary tubes were placed in small plastic tubes and spun at 100 *g* for 6–8 min. The capillaries were removed and cut at the cell-fluid interface. The portion of the capillary containing the cell pellet was then placed in a small circular 20 × 2-mm planchette (Univers Mekaniska Verkstad AB Herrhagsv, 98 Endkede, Sweden) which was then filled with 0.5 ml of the tissue culture medium and sealed with a cover slip. Antigens in varying concentrations were mixed in the culture medium. Triplicate sets of planchettes were used for each antigen concentration and the appropriate controls. Migration of cells from the capillary tubes was allowed to proceed for 18 h at 37°C in an incubator containing 5% CO_2. A fan of migrating cells usually appeared within 2–4 h after incubation and was complete within 12–18 h. The migrating fan of cells in each planchette was then projected on drawing paper in one of two different ways.

In the first method, a Petri dish containing the planchettes was placed in a standard photographic enlarger specially adapted to contain the Petri dish. The light source of the enlarger was decreased sufficiently to permit easy projection of the fan on drawing paper. The second method made use of a Bausch and Lomb microscope (Bausch and Lomb Inc., Rochester, N. Y.) with a projecting attachment by which the migrating fan was magnified 10 times and projected on drawing paper. Irrespective of the method used for projection of the image, these fans were then traced on paper, the tracings were cut out and were weighed on a balance scale. The index of migration was expressed as the following formula:

$$\frac{\text{weights of areas of migration with antigen}}{\text{weights of areas of migration without antigen}} \times 100 = \text{migration index.}$$

The degree of inhibition was calculated by subtracting the migration index from 100.

Statistical analysis. Statistical analysis of the data was calculated by using the Student's *t* test as described in the Handbook of Chemistry and Physics. The degrees of freedom were calculated from the same book (24).

Analytical methods. Rhamnose was determined by the method of Dische and Shettles (25). Quantitative glucosamine determinations were done by a modification of the Rondle and Morgan procedure (26). Quantitative glucose analyses were done by a modified method employing glucose oxidase (Glucostat) available from the Worthington Biochemical Corporation. Ribonucleic acid was determined by the orcinol reaction. Total nucleic acid content and protein concentrations were determined by the absorption of solutions at 260 and 280 nm, respectively, in the Beckman UV

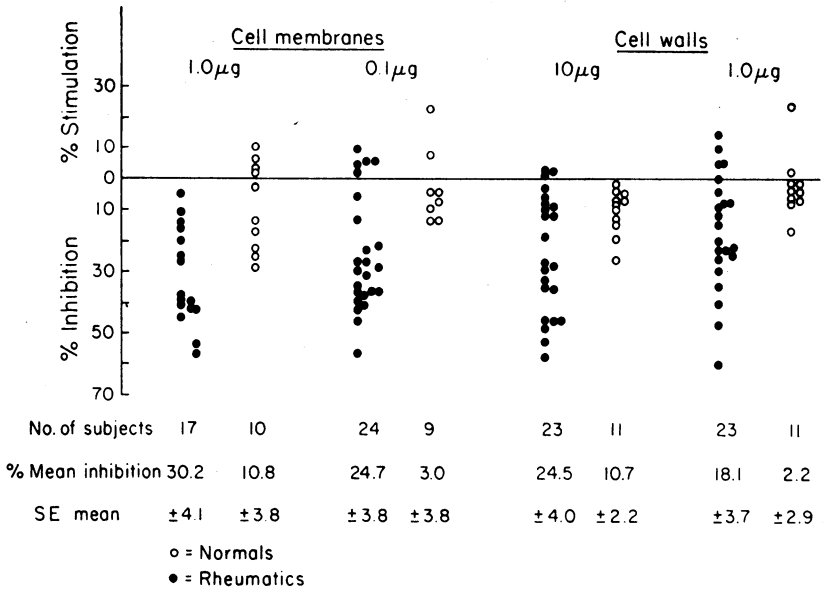

FIGURE 1 Leucocyte migration inhibition values obtained with leucocytes from rheumatic and nonrheumatic individuals after exposure to Group A streptococcal cell walls and membranes. The difference in inhibition to streptococcal membranes in rheumatic vs. normal subjects was highly significant: $P = < 0.01$.

spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Amino acid analysis of the membrane preparation revealed that there was approximately 3–5% *N*-acetyl glucosamine and 3–5% muramic acid present. The determinations of these hexosamines was based on a modification of a previously described method (27).

Serological identification. The serological identification of the strains used in these experiments was confirmed with the capillary tube precipitin test using streptococcal group and type-specific rabbit antisera (28).

RESULTS

Cellular reactivity to streptococcal antigens in rheumatic patients compared to nonrheumatic individuals

To examine the possibility that rheumatic individuals have an increased reactivity to streptococcal antigens, a large group of well-documented inactive rheumatic fever patients were compared to nonrheumatic individuals matched for age and sex. The mean antistreptolysin O (ASO)[1] titers were essentially the same for each group. Migrations were done with at least two concentrations of streptococcal cellular antigens in an effort to determine whether antigen concentration was a significant factor in the cellular reactivity. Fig. 1 demonstrates that, using streptococcal cell wall material as antigen, both rheumatic individuals and normal controls were inhibited to some degree by either 10 or 1 μg concentrations of the cell wall preparation. While the degree of inhibition at both concentrations of cell wall antigens was greater in rheumatic patients as compared with nonrheumatic individuals, the P value of 0.05 for inhibition with 10 μg of cell walls is borderline. The P value of 0.02 for 1 μg of cell walls is acceptable and denotes a significant difference in the degree of reactivity between the two groups. While not shown, the introduction of 0.1 μg of cell walls resulted in no difference between the two groups. This suggests that the antigen responsible for the inhibition is present in lower concentration in the cell wall. In contrast, reactivity to streptococcal cell membranes at either 1.0 μg or 0.1 μg was significantly higher in rheumatic patients when compared to nonrheumatic patients ($P < 0.01$).

Leucocyte reactivity in patients with acute rheumatic fever. The observations that patients recently recovered from a rheumatic attack had the highest leucocyte inhibition values prompted an investigation of the cellular response to streptococcal antigens in a small number of patients with acute rheumatic fever (within the first 2 wk of their disease). A similar group of patients with diagnosed streptococcal pharyngitis were included as controls. These patients were matches for age and sex and were from a similar urban population. All of them had elevated ASO titers, with an average of 600. Clinical signs of pharyngitis, an elevated ASO titer, and the presence of Group A streptococci isolated from throat swabs were the criteria for an uncomplicated streptococcal infection. The clinical and laboratory diagnosis for rheumatic fever was based on the accepted criteria (29) for the disease process. The rheumatic fever pa-

[1] *Abbreviation used in this paper:* ASO, antistreptolysin O.

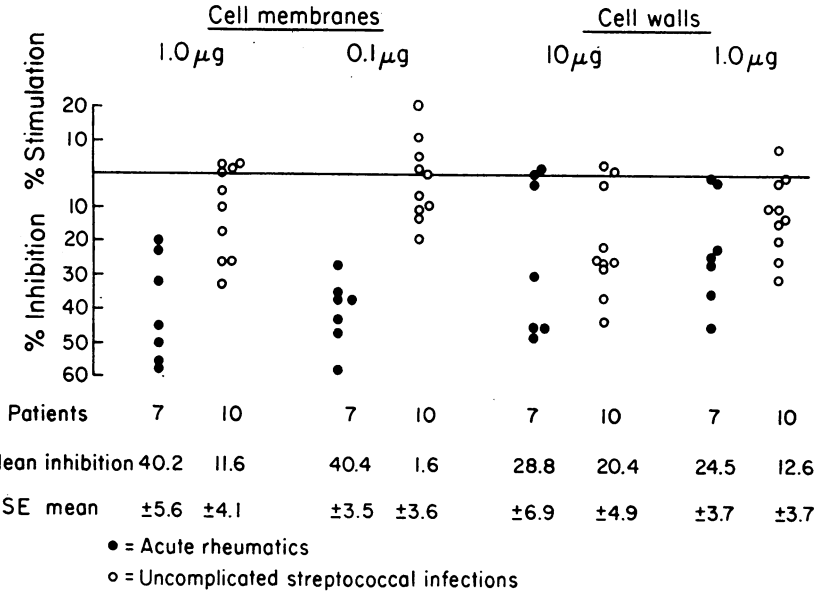

FIGURE 2 Cellular reactivity studies with leucocytes obtained from acute rheumatic fever patients and uncomplicated streptococcal infections. There is marked cellular reactivity of rheumatic leucocytes to streptococcal cell walls and particularly cell membranes.

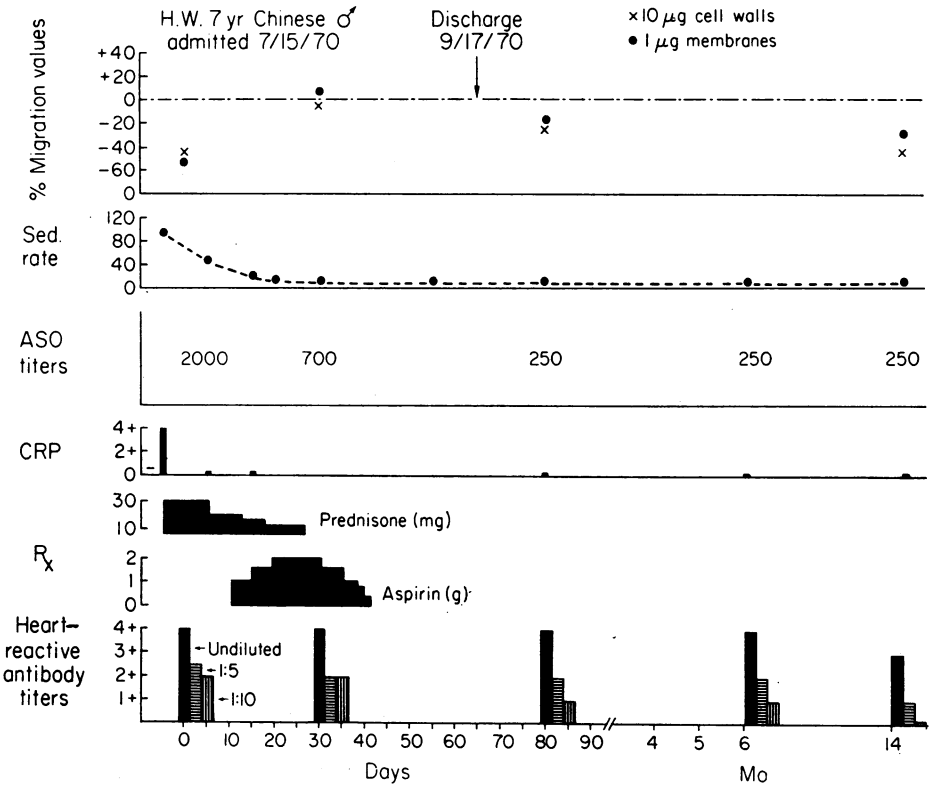

FIGURE 3 A comparison of the serial migration inhibition studies, heart-reactive antibody determinations and therapy in patient H. W. with acute rheumatic fever and rheumatic carditis. Note the change in cellular reactivity both during and after prednisone and aspirin therapy.

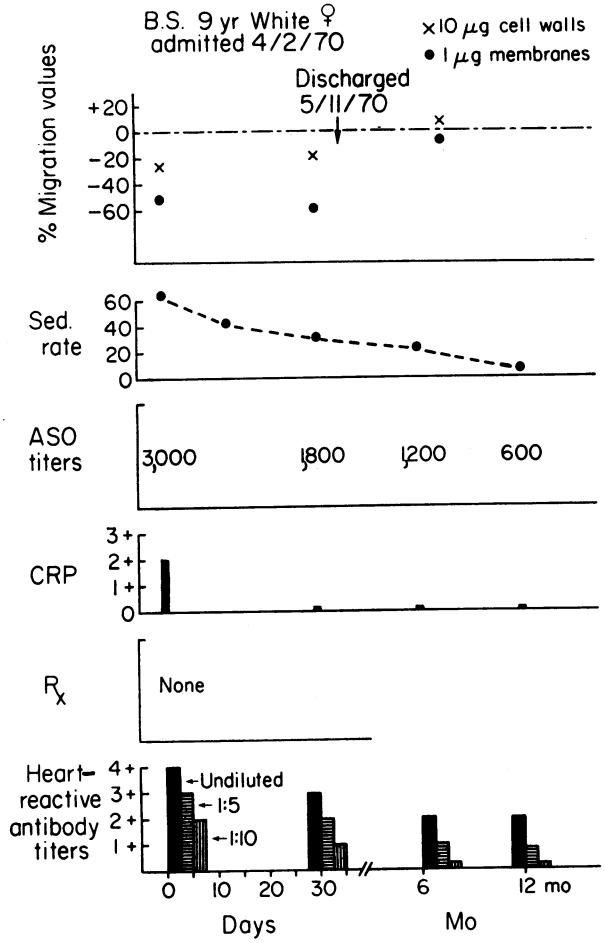

FIGURE 4 Serial migration inhibition results and heart-reactive antibody titers in patient B. S. with acute rheumatic fever who had no evidence of carditis and who was treated with bed rest alone. Migration inhibition values rapidly returned to normal in this patient in spite of persistence of heart-reactive antibody titers.

tients had an average ASO titer of 800. Blood samples were drawn from these patients either at the time of the onset of rheumatic fever and before the institution of anti-inflammatory therapy, or during the convalescent phase (10 days to 2 wk) after the diagnosis of clinical pharyngitis.

While the number of patients with acute rheumatic fever is admittedly small, Fig. 2 demonstrates that patients with acute rheumatic fever were highly sensitive to streptococcal membranes even at 0.1 μg of this preparation when compared to patients with uncomplicated streptococcal infections. The difference in cellular reactivity was highly significant ($P < 0.01$). In contrast, there was no difference in the cellular response of both groups when streptococcal cell walls were used as the antigen which suggests that the leucocytes of all individuals react equally to components of the streptococcal cell walls. Although the degree of inhibition with streptococcal cell membranes (40%) in rheumatic subjects appears to be significantly higher than the inhibition values obtained with streptococcal cell walls (28%), statistical analysis of the data failed to reveal any significant difference between the two antigens ($P = 0.1$).

Repeated determinations of the degree of leucocyte reactivity to streptococcal antigens were carried out in two patients during and after acute illness in order to (*a*) compare the level of heart-reactive antibodies (30) with the migration inhibition values, and (*b*) observe the effect of the treatment schedule on these two parameters of host response to the streptococcus.

Fig. 3 summarizes the results obtained with patient H. W. On admission, the patient had mitral insufficiency with an elevated sedimentation rate of 85 mm/h, C-reactive protein 4+, and ASO titer of 2,000. The heart-reactive antibody titers were elevated. In addition, the migration inhibition values were between 50 and 60%, which shows a high degree of cellular sensitivity to streptococcal antigens. After 30 days of prednisone therapy, the usual laboratory parameters of an inflammatory process had returned to normal as had the migration inhibition values. During a follow-up period of 14 mo, in the absence of any treatment, the migration inhibition values gradually showed more cellular streptococcal reactivity but never to the degree observed during the acute attack. By 14 mo, the heart-reactive antibody values and other laboratory tests were apparently within normal limits, but cellular reactivity to streptococcal cell walls and membranes still persisted.

The second case, B. S. (Fig. 4), illustrates a milder case of acute rheumatic fever, with arthritis and fever as presenting symptoms, in which the only treatment was bed rest. On admission, the sedimentation rate was 60 mm/h, C-reactive protein was 2+, and ASO titer was 3,000, but there was no evidence of carditis either by auscultation or repeated electrocardiogram. Although the migration inhibition test initially showed increased cellular reactivity to streptococcal cell walls and membranes, these values rapidly returned to normal within 6 mo of the acute attack. This occurred at a time when elevated heart-reactive antibody titers were still present in the serum of this patient.

Persistence of cellular reactivity to streptococcal antigens in rheumatic fever patients

Since an analysis of leucocyte inhibition values in acute rheumatic fever patients indicated a significant degree of reactivity to streptococcal membranes, leucocytes obtained from patients who had their attacks 1–10 yr previously were tested with streptococcal antigens in an effort to determine how long this sensitivity to membrane antigens persisted. For the sake of convenience,

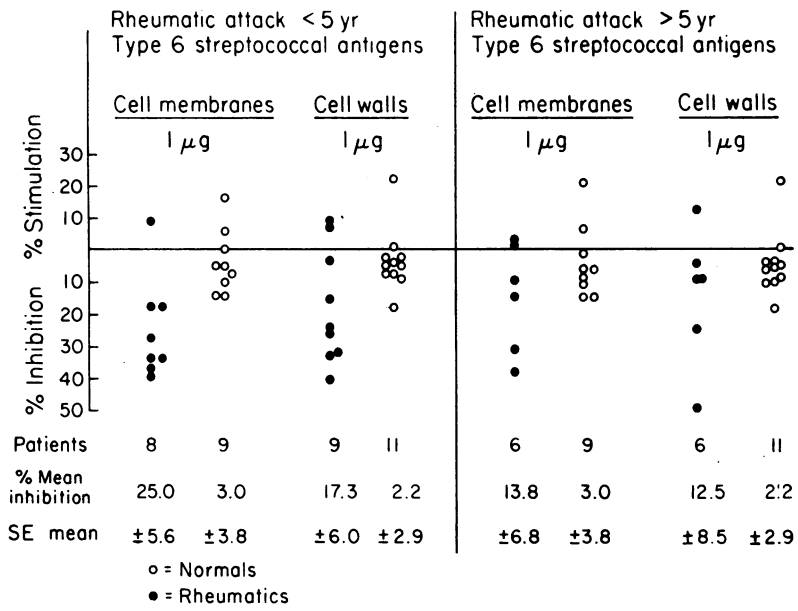

FIGURE 5 A comparison of the cellular reactivity studies to Group A streptococcal cell wall and membrane antigens in rheumatic fever patients who had their attack either less than 5 yr ago or more than 5 yr ago. Sensitivity to streptococcal membrane antigens is maintained in rheumatic subjects up to 5 yr after an initial attack.

these patients were divided into two groups; those patients who had their attack within the past 5 yr and those patients whose attack occurred more than 5 yr ago. An examination of the leucocyte inhibition values in Fig. 5 demonstrates that patients who had their attack less than 5 yr ago continue to exhibit a heightened reactivity to streptococcal membranes even at a concentration of 1 μg of membranes. While there appears to be an increased reactivity to cell wall antigens in rheumatics, the wide variation in response to the cell wall preparations in both groups was reflected in the borderline statistical significance ($P < 0.05$, but > 0.02).

Examination of the cellular reactivity of leucocytes obtained from patients who had their attack more than 5 yr before the study (Fig. 5) indicates that abnormal cellular reactivity to streptococcal antigens is no longer detectable in this group of patients. While an occasional patient reacted strongly to a given concentration of either streptococcal membranes or cell walls, the average inhibition values were the same for this group over two different concentrations of either cell walls or membranes.

Cellular reactivity to streptococcal antigens in rheumatic patients with and without valvular disease

Recent reports by Goldstein, Rebeyrotte, Parlebas, and Halpern (31, 32) indicate that mammalian structural glycoproteins (obtained from mammalian heart valves) contain antigens similar to those isolated from Group A streptococcal cell walls. The shared antigenicity appears to be due to the presence of common *N*-acetyl glucosamine terminal groups in mammalian tissue and the Group A streptococcal carbohydrate. This work is further strengthened by the observations of Dudding and Ayoub (33) that the sera of patients who develop rheumatic valvular disease contain high titers of antibodies to the streptococcal Group A carbohydrate (of which the antigenic determinant is *N*-acetyl glucosamine) and that these antibodies may persist for years after the initial attack. In contrast, some of the patients with non valvular rheumatic fever, as well as patients with rheumatic chorea, also contain Group A carbohydrate antibodies, but the titers fall off rapidly after the acute attack. In view of the known cross-reaction between the structural glycoprotein present in valvular tissue and the streptococcal group-specific carbohydrate, it has been suggested that antibodies to either the structural protein or the streptococcal carbohydrate moiety are responsible for the valvular damage.

Inactive rheumatic fever patients with and without valvular disease were compared (matched for age, sex, and time from original attack) to determine if there was a selective reactivity to streptococcal cell wall structures as compared to membrane antigens. Fig. 6 summarizes the results of these studies, and it is clear that there is no significant difference in the degree of leucocyte inhibition in either group. Although one would

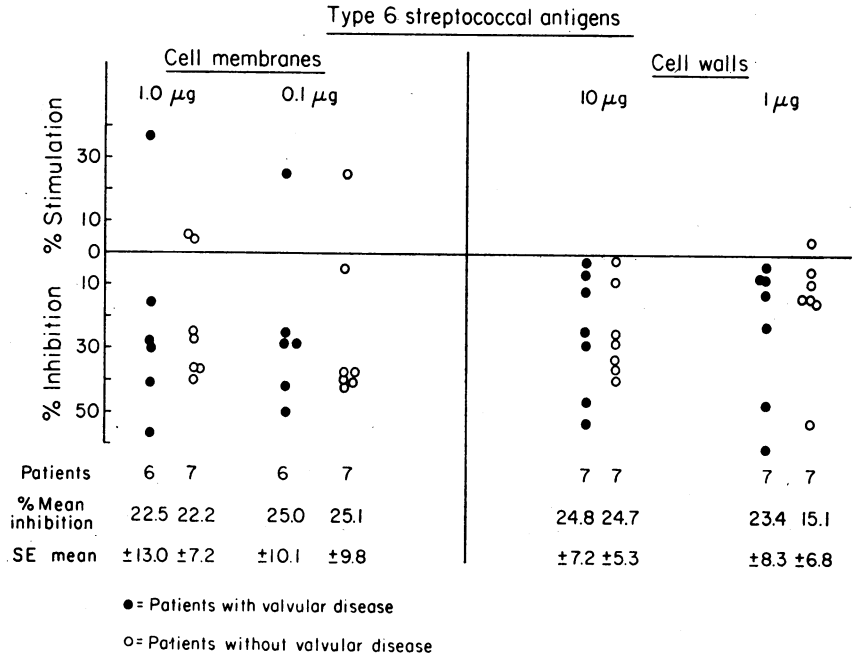

FIGURE 6 Migration inhibition responses to streptococcal antigens in rheumatic subjects who had evidence of rheumatic heart disease compared to patients without evidence of valvular damage. The response to streptococcal cell walls and membranes was the same for both groups.

not expect to see a difference in reactivity to streptococcal membranes, the failure to demonstrate any difference with streptococcal cell walls was surprising and may be due to the limited range of antigen concentrations used. To date, only humoral antibodies, cross-reactive with heart glycoprotein and Group A streptococcal carbohydrate, have been demonstrated in patients with valvular disease (33).

Enzymatic treatment of particulate streptococcal antigens: its effect on leucocyte reactivity in rheumatic subjects

Hypersensitivity studies in both animals and man have stressed the fact that the greatest degree of cellular or skin reactivity to streptococcal antigens was observed when the type-specific strain was used as the test antigen. More recent studies by Beachey et al. (11) and Pachman and Fox (12) have indicated that the type-specific M protein moiety was responsible for the increased cellular reactivity in immunized guinea pigs. These studies suggested that the heightened leucocyte inhibition response to Group A streptococcal antigens might be related to the type-specific M protein surface antigen on streptococcal cell walls. In addition, previous studies involving enzymatic treatment of cell membranes (21) resulted in the release of streptococcal antigens which showed cross-reactive determinants with mammalian muscle tissue. Accordingly, both streptococcal cell walls and membranes were treated with crystalline trypsin (see Methods) for 2 h at 37°C. After this incubation, the cell walls and membranes were washed several times in saline and resuspended to the original concentration. Identical preparations of untreated cell walls and membranes were used as controls. Precipitin reactions or agar immunodiffusion studies with type-specific antisera failed to reveal the presence of M protein in the trypsin-treated cell walls. However, in spite of the absence of type-specific surface proteins on these cell walls, Fig. 7 demonstrates that the cellular reactivity to both preparations was essentially equal. In addition, there was no loss of reactivity of leucocytes from rheumatic patients to trypsin-treated streptococcal membrane antigens.

Cellular reactivity to streptococcal antigens from other hemolytic streptococcal groups

In an effort to determine whether the heightened response to streptococcal antigens was peculiar for Group A streptococcal antigens, cell walls and membranes from Group C and D streptococci were also prepared. Leucocytes obtained from rheumatic individuals and normal individuals were compared with respect to the degree of cellular reactivity to these antigens. Fig. 8 summarizes these studies, and while reactivity to Group A strepto-

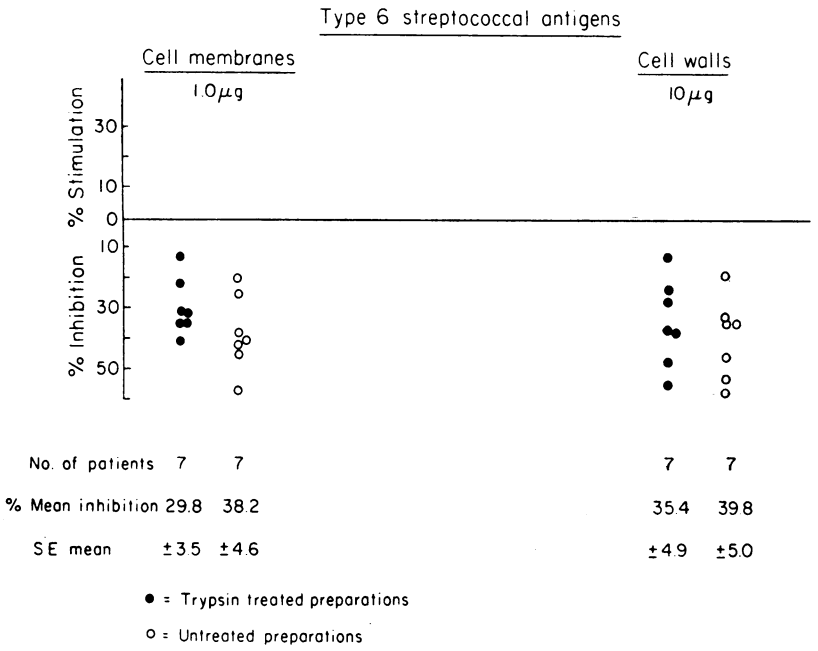

FIGURE 7 A comparison of the cellular migration response in rheumatic subjects to both trypsinized and nontrypsinized Group A streptococcal cell walls and membranes. Enzymatic digestion of both cell walls and membranes made no difference in the cellular response of rheumatic subjects to these antigens.

coccal antigens is heightened in rheumatic individuals, cellular reactivity to Group C and D streptococcal antigens is the same for both groups of patients.

Cellular reactivity to streptococcal antigens in newborn infants

A number of recent reports have stressed the fact that bacterial antigens (34, 35), and in particular streptococcal antigens, may have mitogenic properties. In view of these findings, the cellular response to streptococcal cell walls and membranes was studied in leucocyte populations obtained from newborn infants. Fig. 9 summarizes the leucocyte migration values in 10 newborn infants. It can be seen that the degree of cellular reactivity to both Group A and D membranes, as well as Group A cell walls, is within normal limits in these infants. When the degree of inhibition in newborns was compared to values obtained in normal individuals, no statistical difference between the two groups was observed ($P = 0.1 - 0.8$). The lack of statistical differences between the two groups probably reflects the relative insensitivity of the test system at low inhibition levels. Of greater importance is the fact that these streptococcal antigens did not elicit a nonspecific mitogenic response in newborn infants.

DISCUSSION

Many studies have now demonstrated that the inhibition of migration of human peripheral blood leucocytes in the presence of their specific sensitizing antigen is an accurate in vitro reflection of the state of delayed-type hypersensitivity to that particular antigen (reviewed in 36). It has been shown that only particulate antigens give consistent and accurate results in this direct migration inhibition system (37). Recent studies by Rocklin (38) have shown that a specific factor, leucocyte inhibitory factor, is released by sensitized lymphocytes

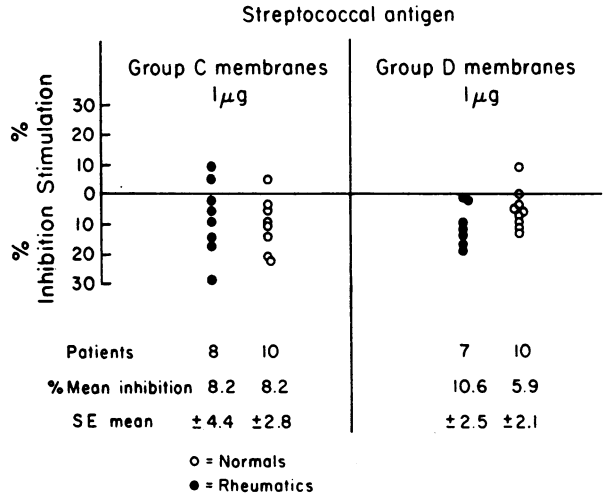

FIGURE 8 The lack of heightened cellular response to streptococcal Group C and D membranes in rheumatic patients and normal control subjects.

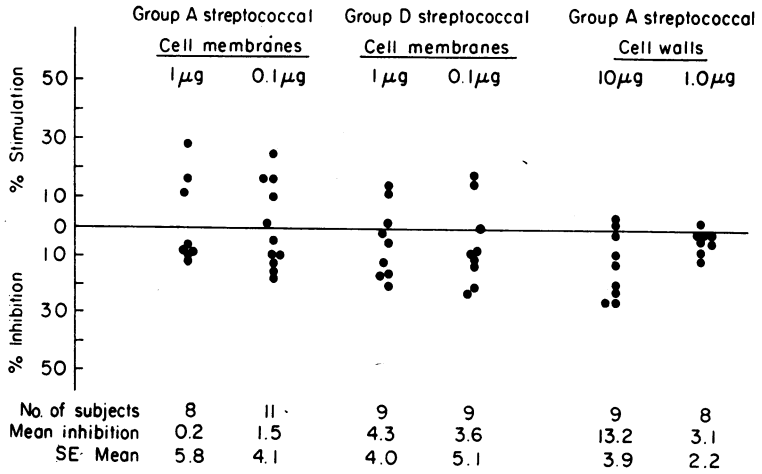

FIGURE 9 A comparison of leucocyte migration inhibition to streptococcal antigens in newborn infants.

after stimulation by specific antigen and that this substance inhibits the migration of human polymorphonuclear leucocytes. He showed that the leucocyte inhibitory factor was distinct from migration inhibitory factor both in molecular weight and in biological activity. He also demonstrated again the correlation between delayed-type skin reactivity and direct leucocyte migration inhibition.

Capillary migration patterns of peripheral blood leucocytes obtained from patients with rheumatic fever and patients with uncomplicated streptococcal infections were studied in the presence of various streptococcal fractions. Because only particulate antigens work in this system, no soluble streptococcal antigens or partially purified wall components, such as M protein, were used directly. While the number of patients studied to date is admittedly small, the following general conclusions may be made. First, there is a heightened cellular reactivity to streptococcal membrane antigens in patients with acute rheumatic fever, even at a concentration of 0.1 μg/ml. This increased cellular reactivity to membrane structures persists in rheumatic patients up to 5 yr after the initial attack without any clinical or laboratory evidence of an intercurrent streptococcal infection or rheumatic recurrence during this period. Afte 5 yr the majority of rheumatic individuals lose this altered sensitivity to membrane structures and respond in a normal fashion to these antigens. In contrast, patients with uncomplicated streptococcal infections with elevated antibodies to streptococcal products, e.g. high ASO titers, react to membrane antigens in a normal fashion. Both groups exhibit a somewhat heightened cellular reactivity to streptococcal cell walls. However, this reactivity drops off quickly in both groups and leaves a high and persistent sensitivity to membrane structures in patients with rheumatic fever only.

Secondly, the specificity of this cellular reactivity for Group A streptococcal membranes was evidenced by the fact that Group C and D membranes elicited normal responses in both rheumatic and nonrheumatic individuals. The possibility that streptococcal cell surface proteins might play a predominant role in the observed sensitivity to streptococcal cell walls was excluded by the fact that trypsin-treated cell walls (extracts of which contained no M protein as determined by the capillary precipitin method) produced the same degree of cellular reactivity as did untreated cell walls.

Thirdly, antigen-antibody complexes do not appear to play a predominant role in the heightened cellular response to streptococcal antigens. The cells used in these experiments had been washed at least five times before use, and *only* heterologous calf serum was used in the test system. While humoral antibody does not appear to play a role in the inhibition of leucocyte migration, cell-bound antibody could still be present on the surface of these antigen-reactive cells and, indeed, may play an important role in the initiation of events in the leucocyte migration inhibition system.

Repeated capillary migration tests in the presence of streptococcal antigens were carried out in only two acute rheumatic fever patients, but the results obtained in these two cases are perhaps worthy of comment. Patient H. W.'s loss of cellular reactivity during aspirin and steroid therapy is probably related to the effect of these drugs on lymphocytes (39, 40). The rapid return and persistence of cellular reactivity to streptococcal antigens in this patient after cessation of therapy contrasts with the relatively rapid disappearance of migration inhibition in patient B. S. This difference raises the question of whether the intensity and duration of a heightened cellular response to streptococcal antigens plays a role in the severity of the attack or in the sus-

ceptibility to rheumatic recurrences. It should be noted that in the two cases presented, we are comparing only overall severity of disease and not the manifestation of arthritis vs. valvulitis. We would postulate that a patient with severe arthritis or chorea might show just such a persistence of cellular reactivity to streptococcal membranes and that this reactivity picture is not specific for carditis only.

Previous studies using streptococcal antigens have left some doubt regarding the specificity of the cellular response. Francis, Oppenheim, and Barile (41) felt the lymphocytic response to streptococcal cellular antigens was specific, since cord blood lymphocytes did not respond to these antigens. In contrast, Keiser, Kushner, and Kaplan (34), using essentially the same type of antigens, noted a definite response of cord blood lymphocytes to these antigens and felt the cellular reactivity was "nonspecific" in nature, akin to the response induced by phytohemagglutinin and other mitogens. These conflicting results may be due to the preparations of antigen used. For example, it has been shown by Taranta, Cuppari, and Quagliata (35) that streptolysin "S" preparations contain a separable hemolytic component and a nonspecific mitogenic component. Studies by Keiser et al. (34) suggest that at least part of the transforming material present in extracts of streptococcal cell walls and membranes may be related to the presence of just such streptolysin S-related materials. Our control studies, including normals matched with the rheumatics studied, and especially the studies on cord blood lymphocytes, showed that we were not dealing with a nonspecific mitogen in our antigen preparation.

Our studies have shown a persistent heightened reactivity of peripheral blood lymphocytes of rheumatics to cell membranes of Group A streptococci. This is of particular interest, as this structure was previously shown to contain antigen(s) cross-reactive with sarcolemma of mammalian heart and smooth muscle of blood vessel walls (21, 42). The nature of the antigen(s) responsible for the observed reactivity is at present unknown. Trypsin treatment of cell walls (and contaminating membrane fragments) did not appear to alter the lymphocytic response to these streptococcal antigens. In addition, the lack of M protein on these treated walls would tend to exclude this protein as an active participant in the reaction. Treatment of streptococcal membranes with trypsin also did not materially affect the cellular response. Whether the lipoprotein portion of the cell membrane plays a role in this reaction has not been ascertained at present, and studies designed to delipidate the membrane are in progress. As mentioned previously, the possibility that circulating antigen-antibody complexes play a major role in the inhibition process appears to be excluded on the basis of the use of heterologous serum in the migration system.

While these results strongly suggest that there is a heightened response to streptococcal antigens in rheumatic individuals, the exact role these sensitized cells play in this disease process remains unknown. The present finding that there is an *abnormal* cellular response to membrane antigens, coupled with previous reports of an abnormal humoral antibody reactive with streptococcal membrane in rheumatics, could argue strongly for an important role of this cell structure in the disease process. The cross-reactive properties of these antigens might conceivably result in autosensitization to tissue antigens producing cytotoxic effects in the tissues of the host. This concept is in agreement with the histological findings of a large number of lymphocytic cells in and near the pathological heart lesions of rheumatic fever (43). The observed humoral response might either prepare the tissues for this autosensitization process or act in conjunction with sensitized cells to produce the damage to the tissues.

Experiments to determine the cytotoxic effect of these sensitized cells for mammalian cardiac cell monolayers and the effect of autologous sera on this reaction are now in progress.

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