STREPTOCOCCAL-INDUCED CELL-MEDIATED-IMMUNE DESTRUCTION OF CARDIAC MYOFIBERS IN VITRO*

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The etiology of poststreptococcal rheumatic carditis is unknown. Various hypotheses (e.g., reviewed by Taranta and Uhr [1] and McCarty [2]) include the direct effect of streptococcal products on tissues, the reaction of anti-streptococcal antibody with streptococcal antigens bound to tissues, and the immune reactions of anti-streptococcal antibodies or lymphocytes cross-reacting with tissue antigens. Experiments clearly demonstrating antigenic relationships between Lancefield group A streptococcal antigens and human or other mammalian heart tissue (3-5) have stimulated a number of investigations implicating autoimmune phenomena in the pathogenesis of rheumatic heart disease. Earliest among these was the work of Cavelti (6) who induced cardiac lesions in rats injected with killed streptococci and heart tissue. He hypothesized that the induction of poststreptococcal rheumatic lesions was a result of streptococcal antigens combining with tissue components, forming new antigenic determinants to which antibodies were induced—the rheumatic lesions presumably resulted from the reaction of these antibodies with tissue.

Additional support for an autoimmune hypothesis was the demonstration by Kaplan and Meyeserian and Kaplan and Svec (7, 8), and confirmed by other (9-11), that sera from a majority of patients with rheumatic fever contain antibodies cross-reactive with mammalian cardiac myofibers. Deposits of gammaglobulin and complement components may be seen in heart muscle of patients who have succumbed to rheumatic heart disease, and heart-reactive rheumatic sera may be absorbed with streptococcal cell walls, thereby abolishing the anti-heart specificity. Zabriskie and Freimer (12) showed that one or more components of the streptococcal protoplast membrane share antigenic identity with cardiac myofibers. A serological relationship between a glycoprotein extracted from heart valves and the group A specific carbohydrate has also been demonstrated, thus implicating valvular rheumatic lesions in the hypothesis of shared antigenic determinants with group A streptococci (13, 14). Cross-reactions between mammalian tissue and streptococci have been thoroughly reviewed by a number of authors (1-4).

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The predominant theme in the autoimmune hypothesis, as demonstrated in the experiments cited above, is the role of antibody in effecting a cytotoxic reaction. However, in many cases of rheumatic heart disease no antiheart antibodies can be demonstrated in the circulation or, at autopsy, at the site of cardiac damage (8, 9). The Aschoff nodule, hallmark of rheumatic myocarditis, is a granulomatous lesion, most likely resulting from a focal inflammatory reaction; neither gammaglobulin nor complement deposits are observed in the Aschoff body (3, 15).

An alternative mechanism involving autoimmune-induced rheumatic myocarditis could involve cell-mediated cytotoxicity (16). Clinical experiments lend some support to this hypothesis. For example, in 1954, Lawrence (17) observed that delayed cutaneous reactivity to group A streptococcal antigens could be transferred in humans with peripheral leucocytes. More recently, Read et al. (18) carried out a survey of cell-mediated responses to streptococcal membrane antigens by peripheral blood leukocytes from rheumatic fever patients. They claimed that cellular reactivity to these streptococcal membrane antigens, measured by the inhibition of migration of peripheral leucocytes in capillaries, was significantly enhanced in these patients when compared with cells from normal subjects or patients with uncomplicated streptococcal infections.

To investigate further the hypothesis that cellular immune responses could play a role in the pathogenesis of rheumatic heart disease, we have devised an in vitro model. The system is based on the widely observed phenomenon that lymphocytes are capable of exerting cytotoxic properties on allogeneic target cells expressing surface antigens to which the aggressive lymphocytes have been sensitized. In the model presented here, embryonic guinea pig myofibers in primary culture serve as specific targets for effector (aggressor) lymphocytes from adult guinea pigs sensitized with group A streptococcal cellular antigens.

Materials and Methods

Streptococcal Antigens. Strains of group A, type 12, and group C streptococci and growth conditions have been described in detail (19, 20). Streptococcal cell walls, probably containing fragments of protoplast membrane and referred to hereafter as broken cells, were prepared in a Braun homogenizer, and separated from dissociated membranes and cell sap by repeated washing and centrifugation (20, 21). Purified protoplast membranes were obtained by treatment of intact streptococci with a group C bacteriophage-associated muralytic enzyme (22). Group A and group C membranes were considered reasonably clean but not entirely free of cell wall material; quantitative determinations showed that the various membrane preparations, on a dry weight basis, contained between 2.4 and 3% hexosamine (23). Membrane suspensions of optical density 0.2 (equivalent to 100 U on the Klett-Summerson colorimeter at 660 nm) were entirely soluble in 2% sodium dodecyl sulfate at 37°C after 5 min. Purified type 12 M protein was prepared according to the procedure of Fox and Wittner (20). Trypsinized cell walls were prepared by suspending the broken cells, after RNAase treatment, in 1% trypsin at 37°C for 18 h (24). Crude acid extracts of streptococci, containing solubilized M protein, group-specific carbohydrate, and unidentified soluble antigens were prepared according to Lancefield and Perlmann (25).

Tissue Antigens. Adult guinea pig heart and skeletal tissue was cut into 0.5-1 cm³ chunks and washed twice with deionized water to lyse the erythrocytes and remove hemoglobin. The minced tissue was homogenized in phosphate-buffered saline¹ (PBS; 0.01 M potassium phosphate, pH 7.0

¹Abbreviations used in this paper: B, bone marrow-derived lymphocytes; CFA, complete Freund's adjuvant; FCS, fetal calf serum; IU, international, unit; MEM, minimal essential medium; PAS, periodic acid Schiff; PBS, phosphate-buffered saline; T, thymus-derived lymphocytes.
in 0.15 M NaCl at 0-5°C in a VirTis homogenizer (VirTis Co., Inc., Gardiner, N. Y.) run at high speed for 15 s, and the homogenate was then poured through a 50 mesh stainless steel wire screen; material not passing through was rehomogenized in PBS. Four to five treatments were sufficient to permit most of the muscle but not the gross connective tissue to pass through the mesh. The pooled muscle homogenates were concentrated by centrifugation, washed twice with cold PBS, and finally resuspended in deionized water and lyophilized.

Heart cell membranes (sarcolemma) from adult guinea pig ventricular tissue were obtained by the procedure of McCollister (26). The procedure consisted of a dissolution of the soluble intracellular proteins in hypotonic buffers followed by repeated washings to obtain the sarcolemmal membranes which were then lyophilized.

**Sensitization and Immunization.** Outbred male and female guinea pigs, 3-4 mo old, weighing 400-500 g were obtained from Scientific Small Animal Farms (Arlington Heights, Ill.). For the induction of cell-mediated reactivities, guinea pigs were injected in the foot pads with 4 mg (dry weight) of streptococcal heat-killed whole cells, broken cells, trypsinized cell walls, M protein or 2 mg of protoplast membranes. These antigens were suspended in PBS and homogenized with equal volumes of complete Freund's adjuvant (CFA) (Bacto, type H-37 Ra, Difco Laboratories, Detroit, Mich.). The antigens in adjuvant were administered in vol of 0.1-0.2 ml in each foot pad. Control animals received the adjuvant emulsified with PBS. A second injection of the same amount of antigen was given 2 wk-3 mo after the primary sensitization; the second antigen (or PBS in control animals) was administered intramuscularly without adjuvant. Animals were sacrificed to obtain lymphocytes 4-7 days after the final injection.

For the induction of hyperimmune sera, the above procedure was carried out with three or four booster doses of streptococcal antigens (without adjuvant) administered subcutaneously at 2-3-wk intervals; antibodies were observed by immunodiffusion with streptococcal acid extracts. This procedure was carried out with guinea pigs and young mature New Zealand white rabbits (supplied by Thompson Industries, Monee, Ill.). In addition to streptococcal antigens, rabbits were immunized with guinea pig sarcolemma (2 mg dry weight) in the above protocol. Absorption of sera was accomplished by mixing equal volumes of sera and packed wet streptococcal broken cells or membranes at 37°C for 1 h followed by incubation for 18 h at 5°C with occasional mixing. The debris was removed by centrifugation, and the procedure was repeated.

**Cell Cultures.** Primary cell cultures of embryonic tissue were prepared from fetuses of guinea pigs sacrificed about 50 days (+ 10 days) through gestation. Homologous tissues (heart, skeletal muscle, liver, or skin) were pooled from litters. The pregnant mothers were anesthetized with ether and exsanguinated before removal of the fetuses. Aseptic surgical techniques were used to secure the fetal tissues which were immediately transferred to a Petri dish containing Eagle's minimum essential medium, spinner modification (MEM-S, Grand Island Biological Co., Grand Island, N.Y.). Hearts from these fetal guinea pigs were dissected free of major blood vessels and atria. The muscle structures from one litter were minced into coarse fragments in 10 ml of MEM-S. The liquid was discarded and the fragments were suspended in 10 ml of Tyrode's solution containing 0.05% trypsin and 0.1% collagenase (ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio) at 37°C. At 30-min intervals the supernatant liquids were collected and the residual tissue fragments were reincubated in fresh enzyme solution two or three additional times to ensure liberation of a sufficient portion of heart muscle cells. To the pooled suspension of cells 5 ml of fetal calf serum (FCS) was added per 25 ml of suspension to inactivate the trypsin. The cells were centrifuged at 280 g for 10 min at 4°C. The pellet was suspended in Medium 199 (Grand Island Biological Co.) containing 10% (vol/vol) heat-inactivated FCS, 100 international units (IU)/ml of penicillin, 100 μg/ml of streptomycin, and 200 U/ml of mycostatin. Portions of cells were stained with 0.1% gentian violet and counted in a hemocytometer. A final concentration of approximately 5 x 10⁶ cells in 0.1 ml were seeded onto each Microtest II tissue culture plate chamber (Falcon Plastics Division of BioQuest, Oxnard, Calif.). After 18 h of incubation at 37°C in 5% CO₂ and 95% air, the culture medium was removed and fresh medium at 37°C was added. Three changes of medium were made before labeling the monolayers with ⁵¹Cr after 48 h. To determine the number of cells at the time of assay, 0.1 ml of 0.25% trypsin in PBS was added to each of 24 chambers for 2 min. The eluted cells were combined, washed once in PBS, and the cell number per chamber was determined in a hemocytometer. Cultures of skeletal muscle, liver, and skin were prepared in the same manner.
Lymphocytes. Peripheral blood lymphocytes were obtained by cardiac puncture exsanguina-
tion from guinea pigs under ether anesthesia. Lymphocytes were isolated from 35 to 40 ml of fresh,
heparinized blood (10 IU/ml sodium heparin; Upjohn Co., Kalamazoo, Mich.) by the Ficoll-
Hypaque (Pharmacia Fine Chemicals, Piscataway, N.J.) density gradient centrifugation tech-
nique of Boyum (27). The blood was layered on top of the Ficoll-Hypaque mixture, centrifuged at
400 g for 40 min and the layer of lymphocytes at the interface was collected. Trypan blue staining
revealed that more than 90% of the cells were viable. The lymphocytes were washed three times in
MEM-S before use in cytotoxicity assays.

Spleens were removed aseptically from adult guinea pigs, connective tissue was removed, and
the spleens were minced through a sterile wire mesh into MEM-S. Erythrocytes were removed by
selective osmotic lysis (27). Spleen cells were washed in 40 ml of MEM-S and passed through a
screen supporting a lens paper filter. The filtrate was centrifuged again. A majority of the
macrophages were removed by incubating the resuspended cells in 20 ml of Medium 199 supple-
mented with 20% FCS in 100 × 75 mm plastic Petri dishes at 37°C under 5% CO2 and 95% air for 45
min. The nonadherent lymphocytes were removed by gently rocking and then transferring for
further incubation. This process was repeated three times. The cell counts at this time revealed a
reduction of approximately one-third to one-half of the original number of cells. Trypan blue
staining showed that more than 90% of the cells were viable. Inguinal lymph nodes were removed
and macerated to obtain lymphocytes as described above.

Thymus-derived (T) and bone marrow-derived (B) lymphocytes from guinea pig spleens were
separated on nylon wool columns according to Julius et al. (28) who developed this procedure for
mouse lymphocytes. Half of the total washed spleen cells from one animal was applied to a column
for T- and B-cell separation. The other half of the total unseparated spleen cell suspension was
retained for comparative studies with the separated cells.

Chromium 51 Release Assay. Monolayers after 48 h in culture were washed three times with
MEM-S before labeling with 1 μCi/0.1 ml of 51Cr (sp act 1 mCi/ml, sodium chromate, Amersham/
Searle, Corp., Arlington Heights, Ill.), added to each chamber 18 h before effector cells were
added. 51Cr was in medium 199 containing 10% FCS. (51Cr was not used after 3 wk from the labeled
date designating the specific activity). Monolayers incorporated at least 5,000 cpm of 51Cr per
chamber and were washed three times with warm medium immediately before use. Spleen, lymph
node, or peripheral blood lymphocytes suspended in MEM-S supplemented with 10% FCS were the
source of effector cells. These were incubated with target monolayer cells for 6 h at lymphocyte-to-
target cell ratios ranging from 1:50 to 1:300. The final vol of liquid in each chamber was adjusted to
0.2 ml with MEM-S. Triplicate cultures were made for each determination. The amount of 51Cr
released from target cells was used as an index of cytotoxicity (29). Labeled monolayers were lysed
with detergent (1% Triton X-100, Calbiochem, San Diego, Calif.) as a measure of 100% 51Cr
released, and monolayers incubated without lymphocytes were counted as background isotope
release. Radioactivity was measured in a Packard Auto-Gamma Scintillation Spectrometer (Am-
bac Industries, Inc., Downers Grove, Ill.). Because viable lymphocytes probably absorbed 51Cr
which was being released by damaged target cells, special care was taken to remove the unat-
tached lymphocytes without further damaging the monolayers. The contents of each chamber was
drawn into a pipette and gently expelled three or four times and 0.2 ml of PBS was used to wash
the monolayers. The supernatant fluid and lymphocytes were collected in 10 × 75 mm disposable
culture tubes and assayed for radioactivity. Percent of specific 51Cr release was calculated as:
\[
\frac{\text{cpm test sample} - \text{cpm background}}{\text{total cpm (released by detergent)}} \times 100
\]
Values presented in Results are the average of three separate culture chambers for each parameter,
each counted three times. Standard deviation from the mean of each assay is shown in the bar
graphs of the figures.

Inhibition of Macrophage Migration. The capillary tube method described by David et al. (30)
was used to assay macrophage migration. Guinea pig peritoneal macrophages were obtained 4
days after intraperitoneal injection of 20 ml of sterile light mineral oil. The cells were washed four
times in MEM-S as recommended and suspended at a concentration of approximately 8 × 10^6 cells
per ml in MEM-S supplemented with 15% fresh guinea pig serum plus 100 IU penicillin and 200 U
Mycostatin per ml. The cells were introduced into a capillary which was sealed, centrifuged, and cut off at the cell-liquid interface. The capillaries were incubated in Mackaness chambers containing MEM-S and the various antigens at a concentration of 25 μg per ml. Triplicate chambers were prepared for each determination and incubated at 37°C for 18 h. Areas of cell migration from the capillaries were measured with an ocular grid in an inverted phase contrast microscope.

**Histological Procedures.** Heart cell monolayers grown on glass cover slips were fixed in situ in 10% formalin in 95% ethanol for 5 min and stained by the periodic acid Schiff (PAS) technique according to the procedure of Pollinger (31).

Indirect immunofluorescence for observing surface antigens of heart monolayers was carried out on primary cultures grown on glass cover slips. The cells were fixed in acetone-methanol (5:2 by vol) for 5 min. Fixed monolayers were incubated with rabbit or guinea pig antisera (diluted 1:5 with PBS) for 2 h at 37°C. After three 10-min washings with PBS, the monolayers were flooded with fluorescein-conjugated sheep anti-rabbit or anti-guinea pig globulin (Sylana Chemical Co., Orange, N.J.) diluted 1:10, at 37°C for 1 h. The stained monolayers were washed twice in PBS and mounted in 25% glycerin in PBS to be examined under a Leitz 513-84 mercury epifluorescence microscope. (E. Leitz, Inc., Rockleigh, N.J.)

Identification of immunoglobulin-bearing B cells in spleen cell suspensions before and after nylon separation of the B- and T-cell populations was carried out by immunofluorescent staining. Cells (approximately 2 x 10⁷) were suspended in 0.25 ml of rabbit anti-guinea pig 7S Ig (Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.) at a dilution of 1:10 in PBS at 4°C for 20 min. The cell suspension was then pelleted in heat-inactivated FCS, centrifuged at 280 g and washed in 2 ml of medium. The pellet was resuspended in 0.25 ml of fluorescein-conjugated sheep anti-rabbit serum (Grand Island Biological Co.) at a 1:20 dilution in MEM-S medium and incubated for 20 min at 4°C. The cells were washed twice in MEM-S and the final pellet resuspended in 0.05 ml of FCS. One drop was placed on a microscope slide, mixed with 25% glycerol-PBS, and examined under a cover slip by fluorescence microscopy.

Results

**Heart Cells in Culture.** 2-day old embryonic guinea pig heart cells in culture consisted of approximately 45% myocytes and 55% fibroblasts. Myocytes were distinguished by the intensity of the PAS stain for the presence of glycogen. They possessed a nearly round nucleus, a granulated cytoplasm, and lacked pseudopodial processes. Fibroblasts contained lipid droplets in the cytoplasm, had a tendency to form pseudopods, and did not stain with the PAS reagent. The myofibers frequently exhibited contractility in fresh (12 h) cultures.

The heart monolayers were shown to retain the antigenic determinants capable of binding antibodies from rabbit or guinea pig anti-streptococcal sera. Indirect immunofluorescence staining of the monolayers showed that gamma-globulin of rabbit sera specific for group A streptococcal broken cells or streptococcal membranes was bound to the monolayers. Moreover, antisera specific for adult heart homogenates or heart sarcolemma also stained the monolayers. These antihuman antibodies as well as the streptococcal heart-specific antibodies could be removed from the sera by absorption with group A streptococcal broken cells.

**Induction of Effector Lymphocytes; Sensitization of Guinea Pigs.** Injection of adult guinea pigs with various group A and group C streptococcal preparations in CFA induced a state of delayed-type hypersensitivity as measured by skin tests carried out 1 or 2 wk after the final sensitization. Whole cells, broken cells, protoplast membranes, and M-12 protein were used as sensitizing agents. Areas of erythema and induration (1–2 cm in diameter) appeared approximately 18 h after intradermal injection of crude acid extracts of whole organisms or
protoplast membranes. Numerous cross-reactions among the various skin-test antigens were apparent. Thus, sensitization was induced, but the procedure gave no indication of specificity or the distribution of antigens among the sensitizing materials.

Macrophage migration inhibition assays with cells from sensitized guinea pigs gave evidence for cell-mediated immune reactivity to streptococcal antigens. Sensitization with streptococcal group A or C whole cells, broken cells, or protoplast membranes induced migration inhibition of peritoneal exudate cells when soluble acid extracts of these antigens were present in the Mackaness chambers. Inhibition values ranged from 60 to 90%. The soluble antigens or tuberculin in the presence of exudate cells from control guinea pigs sensitized with CFA in buffer produced inhibition of about 25%. A considerable degree of cross-reaction was also observed. For example, exudate cells from animals sensitized to group A protoplast membranes were inhibited from migration by extracts of group C membranes or M-12 protein.

**Effector Lymphocyte Cytotoxicity Assays; ⁵¹Cr Release from Target Cells.** Spleen lymphocytes from guinea pigs sensitized to group A streptococcal broken cells were added to ⁵¹Cr-labeled heart cells in increasing lymphocyte-to-target cell ratios. Fig. 1 shows that maximum cytotoxicity was achieved at a cell ratio of 200:1. The apparent decrease in cytotoxicity above this ratio probably represents a depletion of ⁵¹Cr from the supernatant medium due to uptake or binding by the lymphocytes. Base-line controls for this experiment consisted of labeled target cells plus lymphocytes from guinea pigs sensitized with CFA combined with buffer (PBS). From the results of these preliminary experiments lymphocyte-to-target cell ratios of 200:1 were selected for the presentation of data of most of the subsequent assays.

The specificity of target cell susceptibility to lymphocyte cytotoxicity is shown in Fig. 2. Lymphocytes from guinea pigs sensitized to group A streptococcal whole cells or broken cells were significantly more cytotoxic for heart cells than for skeletal muscle or liver cells or skin fibroblasts. In Fig. 2, experiment numbers identify the same batches of fetal tissue from which the various monolayers were cultured. In addition, individual experiment numbers indicate that spleen cells of individual test and control guinea pigs were tested simultaneously as effectors of cytotoxicity.

Spleen lymphocytes proved to be the most efficient inducers of cytotoxicity when compared to lymphocytes from peripheral blood or regional lymph nodes. An example of several experiments comparing these cells from individual sensitized guinea pigs is shown in Fig. 3, employing heart monolayers as target cells.

To test further the hypothesis that group A streptococci contain unique antigens shared by heart tissue, a comparison was made between Lancefield streptococcal groups A and C. It was observed that guinea pig sensitization with group A streptococci (whole cells, broken cells, or protoplast membranes) consistently induced stronger cytotoxic lymphocytes than did sensitization with group C streptococcal fractions or whole cells. These experiments are shown in Figs. 4-6. In these figures experiment numbers indicate individual lots of target cells assayed simultaneously with spleen lymphocytes from donors sensitized
with the various antigens designated in the figures. Thus, regardless of whether whole cells, broken cells, or protoplast membranes were employed as sensitizing agent, group A streptococci appeared to be more efficacious than group C in inducing aggressor lymphocytes with heart-specific cytotoxic properties. In a number of control experiments not shown in these figures, target cells of embryonic liver and skeletal muscle were not significantly destroyed by lymphocytes from animals sensitized to the group C streptococcal antigens. Not all guinea pig sensitized to group A streptococcal antigens in CFA produced cytotoxic lymphocytes despite a high frequency of delayed cutaneous reactions. A summary of the cytotoxicity mediated by spleen cells of the 108 guinea pigs used in these experiments is shown in Table I. A third of the animals sensitized to group A streptococcal antigens had inactive effector lymphocytes. However, relative to spleen cells of guinea pigs sensitized to group C antigens or CFA in buffer, the response to group A streptococcal sensitization was highly significant ($P < 0.001$) in terms of target cell lysis. Neither group A type 12 M protein nor trypsinized cell walls induced cytotoxic lymphocytes.

Separation of spleen lymphocytes into $T$- and $B$-cell-rich populations was carried out to gain further insight into the cytotoxic phenomenon. Unseparated spleen lymphocytes consisted of about 30% $T$ and 70% $B$ cells as determined by
Targets

Heart
Skeletal Muscle
Liver
Skin

% Specific $^{51}$Cr Release

Experiment Number

Fig. 2. $^{51}$Cr release assay. Spleen lymphocytes from guinea pigs sensitized to group A streptococcal whole cells or broken cells were incubated with 2-day old guinea pig heart, skeletal muscle, liver, and skin fibroblast monolayers for 6 h. Lymphocyte-to-target cell ratio was 200:1. Lymphocytes from animals sensitized to whole cells, shaded bars; broken cells, cross-hatched bars; CFA only, white bars. Vertical lines show standard error of the mean from three separate cultures.

Counting immunofluorescent Ig-bearing populations. Macrophages in guinea pig spleen cell suspensions were estimated at about 30% of the total population; the number was reduced to about 10% after adsorption onto plastic Petri dishes. The nonadherent (to nylon) T cells were not immunoglobulin-bearing and were enriched in the effluent population to about 70%. Adherent cells were eluted and this population consisted of about 80% B cells. The B-cell-rich population also consisted of eluted macrophages which did not stain well for surface Ig, although they acquired some fluorescein dye apparently by phagocytosis. Half of the total spleen cells in each experiment was retained without prior treatment on the column. These effector cells were used to measure $^{51}$Cr released before T- and B-cell separation. Control lymphocytes separated in the same manner were from guinea pigs sensitized to CFA only. Heart monolayers and lymphocytes were incubated together for 6 h and the amount of $^{51}$Cr released into the medium was measured. In Fig. 7 it is seen that the T-cell-rich population of spleen lymphocytes sensitized to group A streptococcal broken cells (experiment 16) contained an activity equivalent to spleen cells before separation. The B-cell-rich lymphocytes induced only 5% lysis. Experiments 14 and 20, while demonstrating relatively low activity, nevertheless showed about 50% more activity associated with the T-cell-rich population. This diminished activity may be explained on
Effector Lymphocytes

Spleen   Peripheral Blood   Lymph Node

% Specific 51Cr Release

Experiment Number

1 2 3   2 3   1

Fig. 3. 51Cr release assay. Comparison of guinea pig heart monolayer cytotoxicity induced by spleen, peripheral blood, and lymph node lymphocytes from guinea pigs sensitized to group A streptococcal whole cells. Shaded bars, test lymphocytes; white bars, control lymphocytes. (Procedures as in Fig. 4).

the basis of cell viability. In experiments 14 and 20 the lymphocytes before assay began to take up trypan blue dye, an indication of decreased viability due to the manipulations of cell separation. Lymphocyte-mediated cytotoxicity was obviously sensitive to the viability of T cells. Three transfers of spleen lymphocytes on plastic Petri dishes to remove macrophages (45 min incubation between each transfer) as well as the final incubation time of 45 min, all at 37°C on the nylon wool column probably caused the viability to drop. To improve lymphocyte survival (experiment 16), spleen lymphocytes were passed through sterile glass wool columns three times to remove a majority of the macrophages. Although recovery of the number of lymphocytes was not as good as adsorption on plastic Petri dishes, the cytotoxicity of the eluted T cells improved significantly as seen in experiment 16.

Antibody-Mediated Cytotoxicity. A number of experiments were carried out to determine whether antistreptococcal antibody and complement in the presence or absence of lymphocytes exerted a cytotoxic effect on target heart monolayers. Guinea pig hyperimmune antisera prepared against whole streptococci as well as broken cells exhibited multiple strong precipitin lines in immunodiffusion when reacted with hot acid extracts or cell sap from broken group A streptococci. No attempt was made to identify the soluble antigenic determinants present other than the group polysaccharide and M protein from group A type 12 streptococci. These antisera in dilutions from 1:8 to 1:8,000, with or without fresh guinea pig complement, exerted no significant cytotoxicity for
heart monolayers labeled with $^{51}$Cr. The guinea pig antisera nevertheless were capable of binding to monolayers when observed by indirect fluorescent staining.

Cytotoxic mechanisms described by Perlmann and Holm (32) involving the combined participation of antisera and normal lymphocytes were also investigated. Lymphocytes from unsensitized guinea pigs, at an effector to target cell ratio of 200:1, in the presence of anti-streptococcal sera and guinea pig complement, exerted no significant cytotoxic activity toward the cultured heart monolayers. In addition, anti-streptococcal serum and complement added to sensitized lymphocytes did not augment the cytotoxicity observed with these lymphocytes in the absence of serum.
**Discussion**

The experiments presented here clearly demonstrated that group A streptococcal antigens associated with the protoplast membrane can induce cytotoxicity in guinea pig T lymphocytes. The lymphocytes were capable of specifically destroying allogeneic cardiac heart cells in culture. On the other hand, group C streptococci, serving as nonrheumatogenic streptococcal antigens, induced significantly less lymphocyte cytotoxicity.

Several parameters in the regulation of the target cell cultures are worth noting. Cardiac tissue harvested from guinea pig fetuses too close to term (average gestation is 63 days) yielded an overabundance of fibroblasts in the primary monolayers, resulting in a diminished cytotoxicity reaction with $^{51}$Cr release only 25–30% above background. Immunofluorescent reactions for the presence of streptococcal-specific antigenic determinants on monolayers showed that after 6–7 days in culture binding of antibody was diminished. For this
Guinea Pigs Sensitized with
Group A Whole Cells
Group C Whole Cells

FIG. 6. $^{51}$Cr release assay. Cytotoxicity for guinea pig heart monolayers by spleen lymphocytes sensitized to streptococcal groups A and C whole cells. Shaded bar, test lymphocytes; white bars, control lymphocytes.

### Table I

**Summary of Cytotoxic Effects of Sensitized and Control Spleen Cells on Cultured Heart Cells**

| Sensitizing agent in CFA                  | Number of guinea pigs sensitized | Number of guinea pigs with spleen lymphocytes exerting cytotoxicity on heart cells | $^{51}$Cr released (over background) | >50% | >25% but <50% | <25% |
|------------------------------------------|----------------------------------|---------------------------------------------------------------------------------|-------------------------------------|------|----------------|------|
| Streptococcal group A whole cells, broken cells or membranes | 43                               | 18                                                                              | 11                                  | 14   |
| Streptococcal group C whole cells, broken cells or membranes | 24                               | 1                                                                               | 3                                   | 20   |
| Buffered saline                          | 37                               | 0                                                                               | 4                                   | 33   |
| M-12 protein or trypsinized cell walls   | 4*                               | 0                                                                               | 0                                   | 4    |

* Two guinea pigs sensitized with each antigen.
reason, monolayers were used for target cell reactions 48–52 h after primary culture. Halle and Wollenberger (33) found that the rate of loss of specific muscle differentiated characteristics in culture appeared to be inversely correlated to cellular growth rates. It was also noted that differentiated characteristics of myocytes are lost or there may be an overgrowth of the muscle cell population by fibroblasts in prolonged cultures.

Group A streptococcal antigens cross-reacting with the cultured myocardium were associated with the protoplast membranes. These observations are in line with the findings of Zabriskie and Freimer (12) who observed that antibodies to streptococcal membranes reacted with sections of mammalian heart and skeletal muscle, visualized by immunofluorescence. However, we observed that guinea pig lymphocytes sensitized to group A streptococcal whole cells or broken cells were also cytotoxic for heart cells in culture, and thus, streptococcal antigens associated with cell walls could also be implicated. Kaplan and Meyerserian and Kaplan and Svec (7, 8) claimed their streptococcal-heart cross-reactions were associated with antigens from the bacterial cell wall. The present experiments do not rule out this possibility.

The specificity of both the target and effector cell reactions in these experiments appears to be associated with group A streptococcal sensitizing antigens and cardiac myofibers; group C antigens were considerably less effective as sensitizing agents and cultured tissues such as skeletal muscle or liver were less sensitive targets. Some previous studies have claimed that tissue specificity was
less limited with regard to cross-reactions between streptococci and mammalian tissues. Skeletal muscle was shown to cross-react with antisera of rabbits immunized with group A streptococci (5). In addition to the group A streptococcal cross-reactions, some degree of anti-heart activity was also observed by Zabriskie and Freimer (12) with streptococcal group C membranes.

The specificity of heart autoantibodies in mice immunized with streptococcal membranes was studied by Schwab and Brown (34). Only group A streptococcal membranes, but not cell walls or group C structures were capable of inducing the autoantibody in inbred mice. The antibody reacted specifically with heart sarcolemmal membranes and was inactive against membranes of other tissues including skeletal muscle. Lyampert et al. (10) prepared extracts of guinea pig tissues cross-reacting with anti-streptococcal sera in precipitin reactions. However, there appeared to be a lack of organ specificity in that extracts of skeletal muscle as well as heart showed precipitin lines of identity with anti-group A streptococcal whole cell serum.

Guinea pigs were selected for our experiments because of the high reactivity of this species to bacterial antigens, particularly with respect to cell-mediated responses such as delayed cutaneous hypersensitivity and in vitro leukocyte reactions (reviewed by Bloom [35]). Moreover, we carried out preliminary studies with newborn rat heart primary cultures and spleen lymphocytes from sensitized adult rats. Although rat heart myofibers in culture were convenient and methodologies are well defined (e.g., [36, 37]), spleen or lymph node lymphocytes from sensitized animals were not cytotoxic. Criteria for sensitization of adult rats (either outbred or Lewis strains) with streptococcal antigens were not easily recognized; skin tests for delayed hypersensitivity and macrophage migration assays with peritoneal exudates or peripheral leucocytes were inconclusive in our hands. Friedman et al. (37) cultured rat myocardium as targets for lymphocytes from rat regional lymph nodes. Cytotoxicity was graded microscopically 48 h after the addition of lymphocytes to target cells. Sensitization with heat-killed group A streptococci induced cytotoxic lymph node lymphocytes and the reaction could be inhibited with rabbit anti-rat lymphocyte serum. Some cytotoxic activity was also observed with lymphocytes of rats sensitized with Streptococcus viridans or with Freund's adjuvant alone. We have no ready explanation for the discrepancies between these data and our own experiments with rat tissues.

The cytotoxicity assay employing 51Cr release from target cells in the experiments presented here is expedient for several reasons. The cytotoxic properties of primed lymphocytes manifest themselves on target cells in a relatively short period of a few hours of cell contact (29). The release of 51Cr from damaged cells is interpreted in our system as the direct effect of a target-specific lymphocyte reaction. Unstimulated allogeneic cytotoxicity occurs in this system only after 2 or 3 days of lymphocyte-target contact in cultures; we therefore rule out nonspecific allograft rejection mechanisms in these experiments. In addition, the specific killer effect of lymphocytes sensitized to group A streptococcal antigens, acting strongly on cardiac myofibers, when compared to other tissues, militates against a mechanism of alloantigen (histocompatibility) rejection. Rapaport et al. (38) demonstrated that accelerated graft rejection was induced by circulating antibody specific for group A streptococcal membranes, and Kyogoku (39) was
able to demonstrate that rabbit antisera to sonicated streptococci were cytotoxic for rat heart cells in culture. In our system we have not observed cytotoxic properties in rabbit or guinea pig antisera prepared against whole organisms, broken cells, or protoplast membranes. However, we observed that rabbit antisera to guinea pig heart homogenates, in the presence of guinea pig complement, were cytotoxic to guinea pig cultured heart cells.

A number of human diseases alleged to be of autoimmune origin, including allergic encephalomyelitis, thyroiditis, and myesthenia gravis, have been reproduced in animal models via transferred lymphatic cells (40–42). With regard to rheumatic heart disease, all experiments with laboratory animals have thus far failed to reproduce in vivo myocardial lesions truly mimicking the Aschoff nodule (15, 43). However, the data presented here give some additional credence to the autoimmune hypothesis of rheumatic carditis, particularly with the participation of cell-mediated immune mechanisms leading to tissue injury.

Summary

We have demonstrated that T lymphocytes from the spleens of adult guinea pigs sensitized to group A streptococcal antigens are cytotoxic for cultured fetal guinea pig heart cells. Lymphocyte cytotoxicity, measured by \(^{51}\)Cr release from target cells, was stimulated by sensitization in vivo with group A whole cells, cell walls, and purified protoplast membranes emulsified with complete Freund’s adjuvant (CFA). Sensitization with group C streptococcal antigens in CFA or CFA alone produced lymphocytes with little or no specific cytotoxic activity. Target cells of cultured fetal skeletal muscle, liver, or skin were relatively refractory to effector cell cytotoxicity. The presence of antigenic determinants on the membranes of cultured myofibers, cross-reacting with group A streptococcal cellular antigens, was confirmed by immunofluorescence. These data are discussed in terms of a model for poststreptococcal rheumatic myocarditis in which cell-mediated autoimmune mechanisms may participate.

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