Interaction in vitro between Myocardial Cells and Autologous Lymphocytes and Sera from Patients with Rheumatic Carditis*

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The cytotoxicity of antimyocardial antibodies and peripheral blood lymphocytes from patients with rheumatic carditis on autologous myocardial cells was investigated. Although sera obtained after open-heart surgery from seven out of eight patients with rheumatic carditis were bound to cultures of autologous myocardial cells, they were not cytotoxic. Peripheral blood leukocytes obtained both before and after surgery from one of these eight patients aggregated around and underwent blast transformation when exposed to autologous myocardial cells in vitro, but not after exposure to autologous skin fibroblasts. Only the postoperative leukocytes from another of these eight patients aggregated around autologous myocardial cells in vitro; however, these lymphocytes were also not cytotoxic to autologous myocardium. To investigate the effect of antimyocardial antibodies on myocardial function, antimyocardial antibodies were added to the culture medium of pulsating cultures of mouse myocardial explants. Although antimyocardial antibody from 27 patients with chronic rheumatic carditis and seven postcardiomyotomy patients had no effect on the pulsation of the mouse myocardial cultures, serum γ-globulin from a patient suffering from acute rheumatic fever with myocardial involvement first accelerated and then gradually stopped the pulsation of mouse myocardial explants.

Circulating antibodies reacting with both streptococcal and myocardial antigens and deposits of immunoglobulin and components of complement in myocardial lesions have been detected in patients suffering from rheumatic fever; however, there is no evidence that these antibodies or cell-mediated immunity to these antigens plays any role in myocardial damage.

We have investigated the cytotoxicity of antibodies and peripheral blood lymphocytes from patients with rheumatic carditis on autologous myocardial cells in vitro. The effect of these antimyocardial antibodies on the pulsation of myocardial explants from newborn mice was also investigated. Neither the sera showing antimyocardial activity nor the lymphocytes showing evidence of sensitization to myocardium had any detectable damaging effect on autologous myocardial cells in vitro; however, when added to cultures of mouse cardiac explants, serum from a patient suffering from acute rheumatic fever with carditis first accelerated and then gradually stopped the pulsation of these cultures.

Materials and Methods

Patients and Collection of Tissues

Samples of serum were collected from 100 patients who had suffered from rheumatic fever (diagnosed on the basis of the American Heart Association’s revision of Jones’ criteria) with carditis and had evidence of cardiac impairment at the time of the collection of serum. Samples of serum were also obtained from two patients suffering from acute rheumatic fever with evidence of myocardial involvement. Small pieces of myocardium from the left atrial appendage were collected in Hanks’ balanced saline solution at 4°C, from eight patients requiring open-heart surgery for the sequelae of rheumatic carditis. Serum and lymphocytes (the latter separated from sterile peripheral blood with carbonyl iron) from these eight patients were obtained immediately prior to and between the eighth and tenth days after surgery. All samples of serum were inactivated at 56°C for one-half hour and then stored at −20°C before use. Lymphocytes were used between two to five days after separation from peripheral blood. Serum γ-globulin was obtained by precipitation with a 33 percent saturated solution of ammonium sulphate. Small blocks of tissue measuring about 5 cu mm and containing any suspected lesion in the left atrial appendage were processed for immunopathologic and histologic studies.

Dissociated Human Cardiac Muscular Cells

Cardiac muscular cells were dissociated by incubation with a freshly prepared 0.1 percent solution of trypsin (Nutritional Biochemical Corp.) following the technique of Harary and Farley and were grown in Falcon plastic flasks using a commercially available culture medium (GIBCO Medium 199) supplemented with 20 percent fetal calf serum.
Pulsating Neonatal Mouse Cardiac Explants

Pieces of tissue measuring about 1 cm in diameter were taken from the ventricles of newly born Swiss (Icr) mice were grown as explants on chick plasma coagulated with embryo extract on flying cover slips (1 x 11 cm) placed inside Leighton tubes containing the commercial medium (Medium 199) supplemented with 20 percent fetal calf serum as described previously. The rate of pulsation of the cultures of the explants was counted with a stopwatch after equilibration of the medium at the desired temperature.

Assays for Cytotoxicity

Immediately prior to testing, the cultures were washed and examined with an inverted microscope. A circular area about 1 cm in diameter was delineated with grease pencil on the outer wall of the flask, and the number of cells attached to the flask within this area was counted. Culture flasks were then exposed in triplicate to one of the following: (1) autologous lymphocytes (the ratio of myocardial cells to lymphocytes in a flask is approximately 1:100); (2) lymphocytes plus autologous inactivated serum or serum 

Y-globulin; (3) autologous inactivated serum; (4) equal volumes of autologous inactivated serum and human serum of the AB blood group as a source of complement; and (5) only human serum from the AB blood group.

The final concentration of human serum in each flask was 25 percent. Cytotoxicity was assessed every 24 hours for five days by estimating the proportion of cells permeable to trypan blue and the proportion of cells which detached from the marked area of the Falcon flask. Clustering of lymphocytes around myocardial cells and blast transformation of lymphocytes were also noted. Lymphocytes measuring 12 μ or more before fixation, containing a large nucleus with one or more prominent nucleoli, and revealing basophilia on staining of the relatively ample cytoplasm were identified as blast cells. Peripheral blood lymphocytes from the two patients showing reactivity with autologous myocardium were also assayed for reactivity with autologous skin fibroblasts.

Uptake of thymidine by the cultured cells was estimated by adding 10 microcuries of tritiated thymidine (Methyl-

H3, New England Nuclear Corp.) per milliliter of medium at 24, 48, or 72 hours before fixing the cultures as described elsewhere. For autoradiographic studies, Ilford K3 emulsion was used.

Immunofluorescence

The “sandwich” method was employed, using monospecific rabbit antisera conjugated with fluorescein isothiocyanate against human IgG, IgM, IgA, C3 (B2, C/B3, A), fibrinogen, and albumin, with appropriate controls as described previously.

RESULTS

Monolayers of Human Myocardium

Interaction with Sera and Lymphocytes. Monolayers of human myocardial cells were obtained from 48 to 72 hours after incubation. The cells were mainly of two types: (1) elongated spindle-shaped cells resembling fibroblasts and not showing any striations, and (2) plump fusiform or long strap-like cells showing cross and longitudinal striations (Fig 1). Contrary to the observations of Kasten, at no time did the cells with striation show uptake of tritiated thymidine, although uptake could be seen in an occasional fibroblast. Samples of serum and lymphocytes from eight patients could be tested with autologous myocardial cells. When tested by immunofluorescence, samples of serum obtained after surgery from seven of the eight patients showed sarcolemmal staining with cultures of autologous myocardial cells, as well as cultures of seven other allogeneic myocardial cells, in vitro (Fig 2). Preoperative samples of serum from two of these seven patients also showed sarcolemmal staining, but the preoperative samples of serum from

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Figure 1. Phase contrast photomicrographic of 72-hour-old culture of adult human left auricular appendage, showing elongated strap-like cell with cross-striations (arrow) (original magnification × 435).

Figure 2. Immunofluorescence of viable myocardial cells from patient with chronic rheumatic carditis exposed first to autologous serum and then to fluorescein-treated goat antihuman IgG, showing binding of IgG to surface of autologous myocardial cells (× 350).
the remaining five patients did not reveal any reactivity with myocardium. Preoperative and postoperative samples of serum from one patient did not react when assayed by immunofluorescence either with myocardial cells in vitro or with cryostat sections of human or mouse myocardium. Samples of serum from two patients with acute rheumatic fever showed both sarcolemmal and sarcoplasmic binding of IgG to all of the cultures of myocardial cells tested (Fig 3). None of the 15 samples of serum from persons in normal health (ie, donors from the blood bank) and without any rheumatic stigmata revealed any staining of either cultures or sections of myocardium.

Lymphocytes obtained both before and after surgery from one patient and only the postoperative lymphocytes from another patient aggregated around autologous myocardial cells, but not around autologous skin fibroblasts (Fig 4). Blast transformation was seen in 19 ± 4 percent of the preoperative lymphocytes and 24 ± 5 percent of the postoperative lymphocytes from the first patient between 72 and 96 hours after exposure to autologous myocardial cells in vitro (Fig 5). Less than 3 percent of the blast cells were seen 72 to 96 hours after exposure of this patient’s lymphocytes to autologous skin fibroblasts or after exposure to 20 percent fetal calf serum.8 None of the preparations of sera or lymphocytes was cytotoxic to autologous myocardial cells. Incubation of these myocardial cultures, even with 0.001 mg of streptolysin O activated with cysteinehydrochloride per milliliter of medium, made them permeable to trypan blue.

Immunoglobulins and C3

Localization in Sections of Auricular Appendages. In one of the nine auricular appendages examined,

Figure 3. Immunofluorescence of human myocardial cells in culture exposed first to serum from patient suffering from acute rheumatic carditis and then treated as in Figure 2. Sarcoplasmic staining shows binding of IgC mainly to striations (clearly seen in cells marked with arrows) (original magnification × 480).

Figure 4. Phase contrast photomicrograph taken 24 hours after addition of lymphocytes, showing clustering of peripheral blood lymphocytes (collected before operation from patient with chronic rheumatic carditis) around autologous myocardial cells in vitro. Striations can be seen in elongated fusiform cell (arrow) (original magnification × 350).

deposits of extracellular IgM and C3 could be seen in the periphery of some of the granulomas, and another auricular appendage showed cells containing IgG, IgM, and IgA in and around areas of fibrosis.

Reactivity of Sera with Myocardium

Effect on Pulsating Mouse Myocardial Explants. Examination of acetone-fixed cryostat sections of human (blood group O, Rh−) and mouse myocardium by immunofluorescence after exposure to sera from 100 patients with chronic rheumatic carditis revealed sarcolemmal staining with 19 sera and diffuse sarcoplasmic staining with sera from eight other patients. Twelve of the 19 sera showing sarcolemmal staining had a titer of antistreptolysin O of 100 or more, and all of the four sera showing a titer of antistreptolysin O of 400 or more showed a sarcolemmal type of staining, whereas the titer of antistreptolysin O of the eight sera showing diffuse sarcoplasmic staining varied between 12 and 50. Those sera which reacted with human myocardium also reacted with mouse myocardium, although usually the intensity of immunofluorescence was
greater with human myocardium.

When undisturbed, individual cultures of mouse myocardial explants maintained their rate of pulsation (which varied from explant to explant) at least for 12 hours; however, as also observed by others,14,15 the rate of pulsation of the cultures of myocardial explants increased (1) with a rise in temperature of the medium, (2) after addition to the medium of human serum, ultrafiltrate of human serum, or isotonic glucose solution, and (3) following excessive turbulence in the medium. All studies on the pulsating cultures of myocardial explants were, therefore, carried out at 37°C, avoiding turbulence of the medium and using only γ-globulin (0.5 mg/ml of medium) dialyzed for 48 hours against sterile phosphate-buffered saline solution (0.01 M; pH 7.1). Serum γ-globulin from 15 persons in apparent normal health, from all of the 27 patients suffering from chronic rheumatic carditis who had antimyocardial activity, and from the eight postcardiotomy patients had no effect on the rate of pulsation of eight cultures of mouse myocardial explants tested with each preparation of serum γ-globulin; however, serum γ-globulin from one of the two patients suffering from acute rheumatic fever with carditis consistently accelerated the rate of pulsation of all cultures of mouse cardiac explants, beginning at about ten minutes after addition of the γ-globulin. The cultures showed spasmodic twitching between 50 and 70 minutes after addition of the γ-globulin, after which they finally stopped beating (Fig 6). Initial acceleration followed by spasmodic twitching and stoppage of pulsation of cultures of mouse myocardial explants after addition of this γ-globulin to the culture medium was consistently observed in three different sets of mouse myocardial preparations. Prolonged dialysis of this preparation of γ-globulin against phosphate-buffered saline solution or further purification by chromatography on diethylaminoethyl cellulose did not affect the inhibitory effect of the γ-globulin on pulsating mouse cardiac explants. Immunofluorescence revealed human IgG bound to the surface of the cultures of mouse cardiac explants exposed to this patient's serum γ-globulin. Absorption of this γ-globulin with erythrocytes, spleen cells, or hepatic and renal homogenates from Swiss (Icr) mice did not affect either the inhibitory effect of the γ-globulin on pulsating Swiss (Icr) mouse cardiac explants or the reactivity of the γ-globulin with mouse myocardium when assayed by immunofluorescence, thus showing that the reactivity of the human γ-globulin with mouse myocardium was not mediated by heterophile antibodies.

**DISCUSSION**

The results presented here confirm previous observations on the presence of human myocardium-reactive antibodies in the serum of patients with rheumatic carditis14,16,17 and patients after cardiac surgery4 and also demonstrate blast transformation of lymphocytes after exposure to autologous myocardial cells. Although aggregation of peripheral blood leukocytes around autologous myocardial cells was observed in two patients and blast transformation of an appropriate proportion of preoperative peripheral blood leukocytes18 in only one patient, the myocardium-specific reactivity of the leukocytes from these patients was established by the lack of reactivity of the leukocytes with autologous skin fibroblasts and with fetal calf serum.6,19

The lack of cytotoxicity of antimyocardial antibodies and peripheral blood lymphocytes on autologous myocardial cells in patients suffering from rheumatic carditis suggests that sensitization to myocardial antigens usually does not have a direct role in the pathogenesis of the myocardial lesions. Furthermore, it is possible that sensitization to myocardial antigens in rheumatic fever is, at least in part,18-21 a result, rather than a cause, of cardiac damage. Evidence of similar sensitization to myocardial antigens after myocardial damage is well documented in patients after cardiotomy, after myocardial infarction,4,21 or during the rejection of cardiac transplants.22 An occasional antibody reacting with the surface of myocardial cells, on the other hand, might have detrimental effects on myocardium, as revealed in the system of assay using pulsating mouse cardiac explants. The clinical sig-
nificance of this in vitro phenomenon remains to be evaluated.

Nevertheless, it should be mentioned that most of the sera and lymphocytes which were assayed in this investigation for their reactivity with myocardium were obtained from patients with clinically quiescent rheumatic carditis, and both of the available sera from patients with acute rheumatic fever revealed the presence of antmyocardial antibody. Limitation of materials has not yet allowed us to study the effect of sera, lymphocytes or both from patients suffering from acute rheumatic fever with carditis on autologous or allogeneic myocardium in vitro.

It has been reported that upon exposure to allogenic cardiac homogenate in vitro peripheral blood leukocytes from children with acute rheumatic carditis did not incorporate more tritiated thymidine than similarly exposed peripheral blood leukocytes from nonrheumatic patients; however, the interpretation of this observation is rendered somewhat difficult because, contrary to what is now well established, an allogeneic tissue (ie, heart) homogenate also failed during this investigation to stimulate peripheral blood leukocytes from healthy control persons and rheumatic patients.

The demonstration of immunoglobulins and C3 in some of the Aschoff's granulomas is of interest because we have also observed bound immunoglobulin and C3 in nonfibrotic pulmonary sarcoid granulomas. Tissue-bound immunoglobulin and C3 have also been detected in evolving granulomas of Aschoff's type in an experimental model, however, whether the presence of immunoglobulin and C3 in these lesions is indicative of myocardial damage by immune complexes remains to be elucidated.

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