Autoantibodies and Spleen Cell-mediated Cytotoxicity in Adriamycin-induced Myocardiopathy in Rabbits

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Summary

Spleen cells from rabbits affected by adriamycin-induced cardiomyopathy were specifically cytotoxic for rabbit embryonic heart cells in microcytotoxicity tests. The interactions between “sensitized” spleen cells and rabbit embryonic heart cells were observed by scanning electron microscopy. Sera from these rabbits were shown by immunofluorescence to contain antibodies reactive with rabbit embryonic heart, kidney, and muscle cells. Cytotoxic antibodies were not detected.

These sera contained factors that enhanced the cytotoxicity mediated by “sensitized” spleen cells and made normal spleen cells capable of destroying targets. The detection of these factors and of cytotoxins was dependent on the method of target-effector cells treatment by serum.

Introduction

Adriamycin, a glycosidic anthracycline antibiotic, has shown marked antitumor activity in animals (9, 21) and good results in the chemotherapeutic treatment of leukemias and some solid tumors in man (6, 23). This antibiotic, however, has exhibited undesirable side effects, among which the most severe appears to be an insidious cardiomyopathy affecting patients chronically treated (15). This syndrome appears to occur at a fairly high incidence after a total drug dose of 500 to 600 mg/sq m (5). The syndrome appears immediately or a few months after the last drug administration, the median interval between the end of the therapeutic treatment and the onset of cardiomyopathy being 2.5 months (range, 1 to 6 months). The pathogenesis of AM cardiotoxicity is unknown. A delayed cardiomyopathy, similar to that in man, has been produced in rabbits chronically treated (3, 13).

Pathological changes in the heart were characterized by cell vacuolization and mitochondrial degeneration accompanied by myofibrillar breakdown, lymphocytic infiltration, and myocardial cell destruction.

Some of these findings, namely the sudden onset of the cardiomyopathy and the presence of myolysis and lymphocytic infiltrates, led us to suppose that immunological factors can play a role in the pathogenesis of this cardiotoxic syndrome (12). In other words, we supposed that this myocardial damage can be considered as the final stage of an immune process that started with the release of autoantigenic substances from myocardial cells modified in some way by the drug treatment.

The presence of sensitized lymphocytes and circulating antibodies against human heart has been reported in a variety of myocardial diseases (14).

Evidence that presents drugs as triggering agents of autoimmune phenomena has also been reported in some diseases (24).

The purpose of this study was to investigate humoral and cell-mediated immune response in AM-rabbits. Preliminary data showing the presence of fluorescent autoantibodies and spleen cells “sensitized” toward rabbit myocardial cells in AM-rabbits have been reported recently (12).

Materials and Methods

Experimental Animals. A group of 8 New Zealand White rabbits was chronically treated with AM, as reported previously (3, 4). In brief, animals received 15 biweekly treatment courses, each consisting of daily i.v. injections (0.8 mg/kg) for 3 consecutive days. Rabbits so treated showed a 100% incidence of cardiomyopathy. A separate group of 7 rabbits received 0.85% NaCl solution injections and served as control animals.

Sera. Sera were obtained from 3 control and 3 AM-rabbits sacrificed after the 7th treatment course and from 4 control and 5 AM-rabbits sacrificed after the 15th treatment course. Sera were heat inactivated and stored at −25°C.

Preparation of Spleen Cell Suspension. Rabbits were sacrificed by i.p. injection of sodium pentobarbital, and the spleens were removed aseptically and placed in sterile Petri dishes containing Eagle’s minimum essential medium plus 10% heat-inactivated fetal calf serum. The spleens were teased apart with sterile forceps to liberate the cells. This treatment produced a mixture of single cells, cellular aggregates, and tissue fragments. To obtain a single-cell suspension the mixture was pressed through a fine-mesh wire sieve and left to stand in conical centrifuge tubes at 4°C for 2 min. The cells were then spun down at 2,000 rpm for 10 min and washed once. Cell viability was assessed using the trypan blue dye exclusion technique. Spleen cells not used...
immediately were suspended at a concentration ranging from $10^6$ to $2 \times 10^6$ cells/ml in medium containing 10% dimethyl sulfoxide, frozen by lowering the temperature at a rate of 5°/min until -80° was reached, and stored in liquid nitrogen. The viability of thawed spleen cells was about 80% as judged by the dye exclusion test, and their activity was comparable with that of fresh spleen cells. Spleenic cells from control and AM-rabbits will be referred to, in the text, as normal and sensitized spleen cells, respectively.

Cell Culture System. Primary cell cultures of REH were obtained from 22- to 24-day-old embryos. The great vessels and atria were removed and the ventricles were minced. The cells were released by trypsinization. A cell suspension containing $2.5 \times 10^4$ cells/ml was seeded in plastic flasks (Falcon, Dickinson and Co., Oxnard, Calif.). Primary cell cultures of REK and REM were obtained from the same rabbit embryos by similar methods. Additional cultures, used as controls, consisted of: (a) primary cultures of MEH, obtained by trypsinization of hearts from 18-day-old embryos; (b) WI-38 cells, obtained from F. T. Perkins (National Institute for Medical Research, Holly Hill, London, England); and (c) HeLa cells.

All these cells were grown in monolayer in Eagle’s minimum essential medium with 10% heat-inactivated fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml).

Primary cell cultures were used between the 1st and 3rd passage level after seeding cells in the Falcon No. 3040 microtest plate wells, in which all the microcytotoxicity tests were performed.

Effect of Sera on Rabbit Embryonic Cells. Sera were tested by the microcytotoxicity test (25) with and without guinea-pig complement toward REH cells and by the indirect immunofluorescence test.

For the latter test, rabbit embryonic and WI-38 cells were fixed with acetone for 10 min. Cultures were then air dried, washed once with PBS, and treated with rabbit sera, diluted 1:5, for 30 min at 37°. After 2 washings with PBS the cultures were treated for 30 min at 37° with fluorescein-conjugated IgG fraction from a goat anti-rabbit γ-globulin serum (Cappel Laboratories, Downingtown, Pa.). This antisera had a fluorescein:protein ratio of 3.0 and was used diluted 1:20 in PBS. Cultures were then washed twice with PBS and mounted with buffered glycerol.

Effect of Spleen Cells on REH Cells. Spleen cells were assayed using the microcytotoxicity test (22) with REH cells. Target cells treated with spleen cells were incubated for 48 hr, washed, fixed, and stained (Giemsa). The number of target cells remaining after fixing and staining was used to calculate the cell number at the end of the test. In these cases cytotoxicity was evaluated by 2 different investigators who gave double-blind and independent scores ranging from 1+ (mild cytolyis) to 4+ (marked lymphocytic adherence and cytolyis). The scores given by the 2 investigators were either very similar or identical. In all cases the ratios of effector cells to target cells (100:1, 50:1, etc.) were calculated from the initial number of cells placed in each well. Regression analysis was performed on cell numbers (8 replicates).

The following controls were used in testing the spleen cell effects on REH cells: (a) the cytotoxicity of spleen cells was evaluated on rabbit (REH, REK), mouse (MEH), and human cells (WI-38 and HeLa) in parallel tests; (b) the living spleen cell effect on REH cells was compared to the effect displayed by a parallel preparation after cell disruption by freezing and thawing; and (c) the cytotoxicity of AM alone was tested at different doses on REH cells. AM (and/or its metabolites) content of spleen cells was detected by a fluorometric method (1).

Scanning Microscopy. Cellular changes, induced by spleen cell treatment, were studied using scanning electron microscopy. The cultured cells to be examined were grown on coverslips inserted under the medium in the Petri dishes which were then seeded with normal or sensitized spleen cells. At different time intervals, cultures were washed 3 times in PBS at 37° and then fixed for 30 min at 37° in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3. The coverslips were then placed at room temperature, allowing the aldehyde fixation to take place for 3 hr. After several washings in PBS, samples were postfixed in 1% OsO₄ solution, pH 7.3, at room temperature for 60 min, rinsed in distilled water, dehydrated in graded alcohols and propylene oxide, air dried, and finally coated under vacuum with a thin gold-platinum layer.

An AMR-1000 scanning microscope at 20 kV was used. The pictures were obtained using secondary electrons at an angle of about 30°, using Kodak TXP 120 films.

Effect of Serum on the Interaction of Spleen Cells with REH Cells. All sera were tested by 3 different methods: Method 1, diluted sera and a constant number of spleen cells were mixed just before addition to target cells (REH); Method 2, spleen cell suspensions were added to rabbit serum diluted 1:4 and incubated at 37° for 30 min, the preparations were then diluted with medium, the spleen cells were pelleted, resuspended in fresh medium, and added to target cells; and Method 3, target cell suspensions were added to diluted serum and, after incubation at 37° for 30 min, the preparations were treated as in Method 2 and added to spleen cells. These assays were also performed according to the microcytotoxicity test (22). The ratio of spleen cells to target cells was 50:1. Cells were incubated for 48 hr and then cytolyis was scored as described.

"Lymphotoxin" Assay. The amount of "lymphotoxin" in test and control media was determined by ascertaining the cytolytic activity on human cells (WI-38 and HeLa). Cultures in wells (4 to 6 replicates) were exposed to cell-free supernatant fluids from different representative cultures. Cells were incubated at 37° for 48 hr, fixed, and stained, and the effects were determined.

Rabbit Sera Absorption with Antigen Made from Rabbit Heart. Cells from adult rabbit heart were obtained by trypsinization. Cells were washed 7 times with PBS, pelleted, and suspended in PBS at a 10% concentration. This suspension was sonically treated for 4 min (Biosonic III; Bronwill Scientific, Rochester, N. Y.). The resulting material was used for sera absorption. Sera were diluted 1:2 and mixed with an equal volume of heart antigen. These prepa-

3 The term lymphotoxin is used with the quotation marks because we did not use a pure population of lymphocytes.
RESULTS

Effect of Spleen Cells from AM-rabbits on Cultured Cells. Spleen cells from all AM-rabbits were tested on REH cells. The results are reported in Table 1. The cytotoxic activity was measured by the cell-counting method and by scores given according to the extent of cytolysis when numbers could not be calculated. Results are the average of 8 or 12 replicates. The number of the surviving cells decreased by increasing the number of the spleen cells. Regression analysis showed that these data were fitted by a modified exponential curve (Chart 1).

Spleen cells were then tested on REH, REK, MEH, HeLa, and WI-38 cells at different ratios of effector cells to target cells. Spleen cells from treated rabbits caused cytolysis of REH cells only, while the same cells disrupted by freezing-thawing were ineffective.

At the same time the content of AM (and/or its metabolites) in the spleens was measured and the cytotoxicity of AM at different doses was tested on cultured cells. The minimal concentration of AM found to be slightly cytotoxic for REH cells was 0.01 μg/ml. This eliminated the possibility that the AM content of spleen cells was responsible for the observed cytotoxicity (Table 1).

Guinea-pig complement was not essential for the lysis mediated by spleen cells, nor did it enhance the lysis when added.

Scanning Electron Microscopy. The interaction between REH and spleen cells was studied by scanning electron microscopy. REH cells, incubated for 1 and 5 hr with normal spleen cells, showed an elongated shape and a fusiform prominent body, which probably corresponds to both nucleus and myofilament bundles.

Veil-like cytoplasmic extensions with thin microprojections closely attached to the glass were mainly observed at the cell poles (Figs. 1 and 2). Spleen cells appeared as round cells with the morphology of lymphocytes. These cells were observed in the intercellular spaces, and rarely, lying on REH cell surface.

Advanced cultures (24 to 72 hr) presented REH cells with a flattened body and a surface showing small spherical protrusions, probably corresponding to the cytoplasmic organelles.

Significant differences were noted in REH cell cultures incubated with sensitized spleen cells. At 1 and 5 hr the

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### Table 1

<table>
<thead>
<tr>
<th>Spleen cells from AM-treated rabbits on REH cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degree of cytolysis at the following ratios of effector cells to target cells</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Spleen cells from AM content of the 1st tested dilution of the spleen cell suspension.</td>
</tr>
<tr>
<td>100:1</td>
</tr>
<tr>
<td>Control rabbits</td>
</tr>
<tr>
<td>1*</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>Treated rabbit</td>
</tr>
<tr>
<td>1*</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>No spleen cells added</td>
</tr>
</tbody>
</table>

* Cytolysis was graded as follows: 2+, moderate; 1+, rare cytolysis; -, no cytolysis (12 replicates); numbers, cells left after staining, the average of 8 replicates.

** AM content of the 1st tested dilution of the spleen cell suspension.

* Rabbits sacrificed after the 7th treatment course; the other rabbits were sacrificed after the 15th course.

ND, not done.

* Spleen cells from this rabbit were not cytotoxic for REK, MEH, HeLa, and WI-38 cells.
majority of heart cells presented round lymphocytic elements encrusted on the veil-like cytoplasmic extensions and attached to the cell with short pseudopods (Figs. 3 and 4).

Occasionally, groups of 3 to 4 lymphocytes were clumped together on the REH cell surface. Heart cells showed ultrastructural characteristics different from the control specimens and interpreted as a morphological expression of a cytolytic process. They were extremely flattened, with a granular surface and with occasional smooth depressed areas in the middle of the cell (Figs. 5 and 6).

The cytolytic effect increased with time, being very evident at 48 and 72 hr.

**Sera.** Sera from AM-rabbits were tested for the presence of cytotoxic antibodies toward REH cells, in the presence and absence of complement. None of the sera showed any cytotoxic activity. Sera were also tested on rabbit and human cells for the presence of fluorescent antibody by the indirect immunofluorescence technique. Sera were tested on both normal and AM-treated cells. REH, REK, REM, and WI-38 cells were grown in the presence or absence of AM (0.01 μg/ml) for 3 days and then trypsinized, washed, and seeded on coverslips. The cells were then treated with sera. The results are summarized in Table 2. REH cells treated with sera from the 6 AM-rabbits showed cytoplasmic and nucleolar fluorescence. Some sera gave fluorescence in REK and REM cells. A strong rise in fluorescence was seen when sera were tested on rabbit cells grown in the presence of AM. Controls consisted of (a) rabbit cells treated with control sera; and (b) WI-38 cells grown with and without AM, and treated with control and positive sera. No fluorescence was observed in such controls.

**Effect of Sera on the Cytotoxic Interaction between Spleen Cells and Heart Cells.** REH cells were seeded in wells and immediately treated with 2-fold serum dilutions and with a constant number of spleen cells known to cause a slight cytotoxicity. Cytotoxicity was recorded at the end of the 2nd incubation day by giving scores ranging from 4+ to 1+, according to the degree of cytotoxicity. Three sera from control rabbits and 6 sera from AM-rabbits were tested in different experiments. The results are reported in Table 3.

Cytotoxicity induced by spleen cells from AM-rabbits on REH cells was strongly increased by the simultaneous addition of serum from treated rabbits. To characterize further the activity of the serum on the interaction between spleen cells and targets, we studied the influence of the mode of addition of serum with the 3 procedures described in "Materials and Methods," using the same cell preparation. Factorial analysis of the variance was performed on these results (Table 4). With these experimental conditions, the simultaneous addition of serum and spleen cells to target cells resulted in a very slight enhancement of spleen cell-mediated cytotoxicity (Table 4, Column 7).

Previous treatment of normal, nonsensitized spleen cells with serum from treated rabbits induced a significant ($P < 0.01$) degree of target cell destruction. The incubation of the same spleen cells with normal serum did not induce any cytotoxicity.

Previous incubation of sensitized spleen cells with autologous serum or sera from other treated rabbits resulted in a significant enhancement ($P < 0.01$) of target cell destruction.

Pretreatment of target cells with serum from treated rabbits induced a significant increase ($P < 0.01$) of the cytotoxicity mediated by both normal nonsensitized spleen cells, and sensitized cells.

### Table 3

<table>
<thead>
<tr>
<th>Serum from</th>
<th>Serum cytotoxic titer in cultures treated with a constant number of sensitized spleen cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control rabbits</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treated rabbits</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>6</td>
</tr>
</tbody>
</table>

* Sera alone were not cytotoxic; mixtures of sera and normal spleen cells were not cytotoxic.

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APRIL 1976 1465

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**Table 2**

**Fluorescence elicited by sera from AM-treated rabbits on different cell substrates**

<table>
<thead>
<tr>
<th>AM-treated rabbits</th>
<th>Rabbit embryonic cells</th>
<th>Human embryonic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>REH</td>
<td>AM-REH&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>2</td>
<td>±</td>
<td>++</td>
</tr>
<tr>
<td>3</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fluorescence was graded as follows: –, no fluorescence; ±, slight; +, weak; ++, strong; ++++, bright fluorescence.

<sup>b</sup> AM-REH, AM-REK, AM-REM, AM-WI-38: rabbit and human embryonic cells grown in the presence of AM.

<sup>c</sup> ND, not done.
**Sera Immunoadsorption.** Results from previous experiments indicate that target cells are more susceptible to the action of spleen cells, when preincubated with serum from treated animals. Specific antibodies were detected by the indirect immunofluorescence test. To check further for the presence of antibodies and to test which role, if any, they play in the cytotoxic phenomena, 2 sera from AM-rabbits were absorbed with heart antigen. After immunoadsorption, sera were tested for fluorescent antibodies and for enhanced cytotoxicity. After absorption the sera completely lost their activity in the immunofluorescence test with REH cells and their enhancing effect on the cytotoxicity mediated by normal and sensitized spleen cells.

"Lymphotoxin." Supernatants from cultures of (a) spleen cells incubated with REH cells and (b) spleen cells incubated with serum and REH cells for 48 hr were assayed for cytotoxicity against WI-38 and HeLa cells.

No cytotoxins were detected in supernatants from the cultures treated with spleen cells alone or from the cultures treated simultaneously with spleen cells and serum. Cytotoxicity was observed when treatment was performed with the supernatants from cultures where spleen cells or target cells had been pretreated with serum, the cytotoxicity being much stronger in the latter case (Table 5).

**DISCUSSION**

Spleen cells from rabbits displaying myocardiopathy induced by chronic treatment with AM were cytotoxic for REH cells grown in vitro. Nonsensitized spleen cells obtained from normal rabbits, incubated with heart cells, did not destroy target cells. Cellular changes, induced by sensitized spleen cell treatment, were studied using scanning electron microscopy. After a 1-hr incubation, the majority of REH cells showed lymphoid elements closely attached to their surface. This phenomenon was followed by severe modification of the cell structure, interpreted as a morphological expression of a cytolytic process which progressively increased with incubation time.

The tissue destruction reported in these experiments suggests that spleen cells from AM-rabbits have a high degree of cytotoxic specificity. When sensitized spleen cells were incubated with REK, MEH, and human cells, no target lysis was observed.

The cytotoxicity displayed by sensitized spleen cells could not be due to AM contained in the spleen, since the drug was present in a concentration that was not cytotoxic for embryonic heart cells.

Moreover, cytotoxic activity was displayed only by living

**Table 4**

Enhancement of spleen cell cytotoxicity by sera from AM-treated rabbits: determination of average number of REH target cells remaining after the culture period

<table>
<thead>
<tr>
<th>Rabbit donor</th>
<th>Cell control, no spleen cells or serum added</th>
<th>Spleen cells, no serum added</th>
<th>Pretreated spleen cells</th>
<th>Pretreated target cells</th>
<th>Mix and add</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>Control</td>
<td>1025 ± 141</td>
<td>994 ± 100</td>
<td>867 ± 192</td>
<td>989 ± 183</td>
</tr>
<tr>
<td></td>
<td>Control-Treated-A</td>
<td>776 ± 95</td>
<td>674 ± 91</td>
<td>648 ± 92</td>
<td>654 ± 84</td>
</tr>
<tr>
<td></td>
<td>Control-Treated-A</td>
<td>776 ± 95</td>
<td>674 ± 91</td>
<td>648 ± 92</td>
<td>654 ± 84</td>
</tr>
<tr>
<td></td>
<td>Control-Treated-B</td>
<td>558 ± 36</td>
<td>503 ± 29</td>
<td>271 ± 64</td>
<td>495 ± 54</td>
</tr>
<tr>
<td></td>
<td>Control-Treated-C</td>
<td>375 ± 57</td>
<td>288 ± 34</td>
<td>197 ± 33</td>
<td>302 ± 90</td>
</tr>
<tr>
<td></td>
<td>Control-Treated-C</td>
<td>375 ± 57</td>
<td>288 ± 34</td>
<td>197 ± 33</td>
<td>302 ± 90</td>
</tr>
</tbody>
</table>

* See text for details.

**Table 5**

"Lymphotoxin"-induced target cell lysis

<table>
<thead>
<tr>
<th>Rabbit donor</th>
<th>Decrease in WI-38 number (%) caused by supernatants from REH cultures incubated with spleen cells and serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>Spleen</td>
</tr>
<tr>
<td>--------------</td>
<td>--------</td>
</tr>
<tr>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td>Control</td>
<td>Treated</td>
</tr>
<tr>
<td>Treated</td>
<td>Control</td>
</tr>
<tr>
<td>Treated</td>
<td>Treated</td>
</tr>
</tbody>
</table>

* Average of 4 cultures as determined by cell counting. The degree of lysis was determined using the expression:

\[
1 - \left( \frac{\text{No. of WI-38 cells incubated with stimulated supernatant}}{\text{No. of WI-38 cells incubated with nonstimulated supernatant}} \right) \times 100
\]
spleen cells since disruption by freezing-thawing abolished cytotoxicity.

The observation that sera from AM-rabbits did not show cytotoxic antibodies, but showed fluorescent antibodies, prompted us to design experiments to ascertain whether antibodies can play a role in the interaction between spleen cells and heart target cells.

Humoral antibodies leading to the phenomenon of enhancement, and humoral blocking factors that interfere with lymphocyte cytotoxicity have been demonstrated in the sera of animals and patients by 3 general methods: simultaneous treatment of target cells with serum and spleen cells, and preincubation of serum either with target cells or with spleen cells. Therefore we analyzed the activity of the serum on the interaction between spleen cells and target cells following these 3 procedures.

Preincubation of target cells with sera from treated animals made normal spleen cells capable of destroying target cells and enhanced the cytotoxicity mediated by sensitized spleen cells. These results demonstrate that we are dealing mainly with 2 different cytotoxic pathways, namely, CMC and ADCC, concomitantly present in AM-rabbits and capable of acting simultaneously in the in vitro destruction of embryonic heart cell. Presumably, other mechanisms are involved in such a phenomenon. In fact, with normal spleen cells and "immune" sera, it was also possible to obtain cytotoxicity by first exposing lymphoid cells to serum and adding them to target cells after washing. It is possible that the cytotoxic cells in these reactions were macrophages, since there is good evidence that macrophages have receptor sites for cytophilic antibodies (2, 10), whereas lymphocytes have low affinity for such antibodies not reacted with antigen. It is therefore unlikely that lymphocytes have gained their cytotoxic potential by mere antibody adsorption. However, it is still possible that they became cytotoxic for having adsorbed antigen (heart antigen)-antibody complexes (19). Perrelli et al. (20) have suggested that the uptake of antigen-antibody complexes may transform "non-immune" lymphocytes into specific effector cells capable of destroying antigenic target cells.

The search for cytotoxin in the supernatants of representative cultures gave unexpected results. We could not detect any cytotoxic factor in the supernatants of REH cultures incubated with lymphoid cells for 48 hr, whereas this factor could be easily detected in the supernatants of cultures where spleen cells or targets have been pretreated with immune sera. These findings are at variance with the other reports on CMC in which cytotoxins have been consistently reported. One reason for our failure in detecting cytotoxins in our CMC tests could be the low, although significant, degree of cytotoxicity obtained with spleen cells in the absence of serum, and the sensitivity of the system used to detect cytotoxins [not very great if compared with 51Cr-release technique coupled with the use of very sensitive target indicators (16)]. On the other hand, these technical limitations did not prevent us from detecting cytotoxins in ADCC, a quite unexpected finding, in view of the negative results reported in different experimental systems (17, 18, 26). To our knowledge there are no reports on ADCC in rabbits, and it can well be that this species behaves differently. With these considerations in mind, these discrepancies may be more apparent than real.

The presence of antibodies directed toward heart antigens was detected by immunofluorescence in all the AM-rabbits. We do not know whether these antibodies are the same as those mediating ADCC; further work is required to elucidate this point. The possibility that the antibodies could be directed toward the fetal antigens of the REH cells was ruled out by the results obtained with immuneabsorption. Sera absorbed with antigen made from adult normal rabbit heart did not give any fluorescence when tested on REH cells, lost the capability to enhance the cytotoxicity mediated by sensitized cells, and did not induce mediation of cytotoxicity by normal spleen cells.

The origin and nature of the antigen(s) as well as the mechanism of their emergence can only be speculated. The activation of lysosomal enzymes triggered by the intracellular acidosis brought about by the anthracycline-induced mitochondrial impairment (8, 11) could produce material endowed with autoantigenic properties, according to the unifying hypothesis of Laufer (14). The rise in the immunofluorescence test response by cells cultivated in the presence of AM can be interpreted as being in favor of the role played in this model by some kind of drug-induced new antigen(s) (7).

Alternatively, AM could provide antigenic determinants to this "new" antigen making a substrate more suitable for the detection of the corresponding autoantibodies.

ACKNOWLEDGMENTS

The authors thank O. Bellini, for valuable technical cooperation with scanning electron microscopy, and Dr. C. Pollini, for her assistance with statistical analysis.

REFERENCES


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Fig. 1. REH cells incubated for 1 hr with normal spleen cells. The elements present a fusiform body with veil-like cytoplasmic extensions at the cellular poles and thin processes closely adherent to the glass. × 1000.

Fig. 2. REH cells incubated for 5 hr with normal spleen cells. A veil-like cytoplasmic extension at high magnification. The cell surface shows small granular protrusions probably corresponding to cytoplasmic organelles. × 2000.

Figs. 3 and 4. REH cells incubated for 1 hr with sensitized spleen cells. A lymphoid element adheres with short pseudopods to the cytoplasmic extensions of a heart cell. × 3000.

Fig. 5. REH cells incubated for 5 hr with sensitized spleen cells. A heart cell shows a granular surface with a depressed central area; a lymphoid element (arrow) is attached to the cellular edge. × 1000.

Fig. 6. REH cells incubated for 5 hr with sensitized spleen cells. A group of lymphoid elements encrusts a heart cell, which shows severe cytolytic changes. × 1500.
Autoantibodies and Spleen Cell-mediated Cytotoxicity in Adriamycin-induced Myocardiopathy in Rabbits

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