Decreased Reactivity of Lymphocytes in Mixed-Leukocyte Culture from Patients with Rheumatic Fever

By Richard D. Lueker, M.D., and Ralph C. Williams, Jr., M.D.

SUMMARY
The purpose of this study was to evaluate cellular immune responsiveness in patients with acute rheumatic fever (ARF). Peripheral blood lymphocytes were obtained for culture from patients who had ARF within the past 2–3 months. No patient was receiving steroids at the time of the study. Peripheral lymphocytes were also obtained from normal control individuals.

Lymphocyte cultures were maintained for 7 days in minimum essential medium with 10% fetal calf serum in a 5% CO₂ environment. Cellular responsiveness was checked concomitantly with pokeweed mitogen and/or phytohemagglutinin. Mixed-lymphocyte cultures were studied between patients with ARF and between patients with ARF and normal controls. Normal responses were arbitrarily defined as a threefold increase over baseline counts.

ARF cells were capable of stimulating other ARF cells in only three of 14 instances and were able to stimulate control cells in only three of 11 studies. Conversely, ARF lymphocytes were capable of being stimulated by control normal cells in five of 10 experiments. Thus decreased cellular responsiveness and abnormalities in cellular immunity are present in many patients with ARF, since lymphocytes from patients with ARF are usually incapable of stimulating normal or other ARF cells. However, ARF cells are capable of being stimulated by normal control cells in 50% of studies performed.

CLOSE HISTOLOGIC inspection of the microscopic cardiac lesions in acute rheumatic fever, such as areas of acute myocarditis or Aschoff bodies themselves, often shows prominent cellular infiltrations with small and medium-sized lymphocytes. It is surprising, therefore, that more attention has not been directed to a study of circulating lymphocytes in individuals afflicted with acute or subacute rheumatic fever. There is indeed a voluminous literature dealing with various humoral antibody responses to streptococcal products or antecedent streptococcal infection in such patients but until recently little work has been reported on abnormalities or anomalies of behavior among peripheral lymphocytes in patients with rheumatic fever. Several authors have examined lymphocyte responses to various soluble streptococcal cellular products using lymphocyte culture or technics measuring production of migration-inhibition factor (MIF) as an in vitro correlate of cellular immunity.

The present study was aimed at a direct examination of in vitro lymphocyte reactivity using the technic of one-way mixed-leukocyte culture originally described by Bach et al. The results reported here appear to indicate a marked degree of impaired cellular reactivity in lymphocytes obtained from patients with acute rheumatic fever; our findings indicate that circulating peripheral blood lymphocytes from such patients are thus functionally impaired.
abnormal and thus may be implicated directly or indirectly in the pathogenesis of the rheumatic state itself. It is also conceivable that the functional abnormality of peripheral blood lymphocytes may be a nonspecific effect associated with the disease.

Materials and Methods

The observations recorded here were performed using the one-way mixed-leukocyte stimulation technic in which lymphocytes from two donors are cultured together for 7 days. One of the lymphocytes of each pair (stimulator) is blocked by preincubation with mitomycin-D so that it is incapable of division and labeled-thymidine uptake, although it still retains its antigenic capacity to stimulate cells of the other member of the cell-culture pair. Thus, when two-way cultures are performed, lymphocyte ability both to stimulate or respond to the other member of the cell-culture pair can be studied separately. This primary method was chosen as a sensitive technic which could be used to study minor abnormalities of cell interaction not detectable using conventional lymphocyte stimulation as with plant mitogens or various streptococcal products.

In mixed-leukocyte cultures, $1 \times 10^6$ leukocytes/ml were used as responding cells and $1 \times 10^6$ leukocytes/ml as the stimulating antigen. Each experiment was performed in triplicate and cell mixtures incubated at 37°C in a 5% CO₂ atmosphere. Harvesting of control cells stimulated with pokeweed mitogen was performed on the third day and on the seventh day of culture for the mixed-leukocyte reactions. Controls for the reactivity of all cell samples studied included their thymidine incorporation after stimulation with plant mitogens, principally pokeweed (0.15 ml of a 1:100 dilution of standard stock solution obtained from Grand Island Biological Company). In most instances rheumatic fever (RF) patients' leukocytes were studied in mixed-leukocyte culture with both normal and other patients with rheumatic fever. The ability of RF leukocytes to stimulate and to respond to both other RF leukocytes and those of normal nonrelated donors was thus recorded. The stimulus provided between mitomycin-treated stimulating cells and unblocked responding leukocytes varies with the antigenic disparity in HL-A loci and possibly other cell surface antigens present on the admixed cells. In all instances here studied, cell pairs tested in MLC were disparate in at least one major HL-A locus.

Positive reaction in the MLC were arbitrarily scored as being threefold incorporation of labeled thymidine above baseline counts. Baseline was considered to be the radioactivity or counts recorded in cells alone cultured in the presence of autologous cells treated with mitomycin.

Some attention was also directed at tests for possible blocking antibodies in plasma of patients with rheumatic fever. Sera were tested for cytotoxicity against a panel of normal human peripheral blood lymphocytes using the microdroplet technic of Terasaki et al. In addition, 0.2-ml aliquots of RF plasmas were added to mixed-leukocyte culture utilizing two normal donors and mixed cultures subsequently processed after 7 days. In no instance was any significant plasma-blocking effect thus detected in the 10- to 20-fold stimulations recorded. In addition, several experiments used MLC reactions between two patients with acute RF to which aliquots of individual-patient plasma (0.2 ml) were added. No differences were recorded when such culture results were compared with those to which no additional RF plasma had been added.

Patient Material Studied

Patients with rheumatic fever were derived from our clinical material at the Bernalillo County Medical Center, Presbyterian Hospital, Lovelace Bataan Hospital, Albuquerque, New Mexico, and from several U. S. Public Health Service Hospitals on the Navajo Indian Reservation. All patients classified as ARF satisfied the Jones criteria or showed recent flagrant clinical evidence of active carditis and were studied within 2-8 weeks after the onset of their acute rheumatic fever episode. No patient included in this study had received corticosteroids; however, several were receiving aspirin or penicillin at the time of collection of peripheral-blood leukocytes. In three patients strong reaction for serum antibody to myocardium was demonstrated through the courtesy of Dr. John Zabriskie, Rockefeller University, New York, New York. This antibody has been strongly correlated in previous reports with the presence of clinically significant attacks of acute rheumatic fever.

Results

Lymphocytes from patients with acute rheumatic fever (ARF) were capable of stimulating other ARF cells in only three of 14 instances (table 1). Lymphocytes from ARF patients were capable of stimulating control lymphocytes in only three of 11 studies. Conversely, ARF cells were capable of being stimulated by control cells in five of 10 instances. Normal lymphocytes responded to nonrelated normal stimulating cells in 25 of 25
Table 1

<table>
<thead>
<tr>
<th>Cells</th>
<th>Stimulation (no.)</th>
<th>No stimulation (no.)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARF*† cells + ARF cells</td>
<td>3</td>
<td>11</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ARF* cells + normal cells†</td>
<td>3</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Normal cells* + ARF cells</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Normal cells* + normal cells</td>
<td>25</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Stimulating cells treated with mitomycin O.
†ARF cells — rheumatic fever lymphocytes.
‡Normal control lymphocytes.

In parallel control experiments. A variety of reactions was observed in the ARF cells, namely that their ability to stimulate and their ability to respond to stimulation did not parallel each other. Examples of representative data are shown in figures 1 and 2.

In figure 1, it can be seen that cells from patient Arch. with ARF could not stimulate other ARF cells from Mur. and could not stimulate normal control cells from Rab. Although ARF cells from Arch. could not respond to stimulation by other ARF (Mur.) cells, they were capable of stimulation by normal control cells (Rab.). ARF cells from Mur. were incapable of stimulating other ARF cells (Arch.) and normal cells (Rab.). Mur. cells were incapable of responding to stimulation from other ARF cells (Arch.). Contrary to patient Arch., cells from patient Mur. were not capable of stimulation by normal cells.

In summary, ARF cells were incapable of stimulating or responding to stimulation from other ARF cells. In one study ARF cells were able to respond to stimulation by normal cells, and in the other they were not. ARF cells could stimulate normal cells in one instance but not in the other. Finally, positive stimulation (not shown in fig. 1) was obtained using plant mitogens (PWM) with all test cells confirming their potential for reactivity and viability.

**Figure 1**

Results in patients Arch. and Mur. Abbreviations: ARF = acute rheumatic fever; RF Baseline = control baseline count in unstimulated ARF cells; RF Stimulating = count resulting from mixed-leukocyte reaction using ARF mitomycin-treated cells as stimulating cells; Normal Stimulating = count resulting from mixed-leukocyte reaction using normal control mitomycin-treated cells as stimulating cells; Normal Baseline = control baseline count in unstimulated normal control cells.

**Figure 2**

Results in patients Yaz. and Gal. Abbreviations same as in figure 1.
In figure 2, ARF cells from patient Yaz could not stimulate other ARF cells from Gal. and could not stimulate normal control cells (Lue.). Furthermore, ARF cells from Yaz could not respond to stimulation by either ARF cells from Gal. or by normal cells from Lue. ARF cells from Gal. were capable of stimulation by ARF cells from Yaz, but not by normal cells (Lue.). These data show that ARF lymphocytes may stimulate other ARF lymphocytes and yet be unable to respond to stimulation by the same cells. Further, although ARF cells may not be stimulated by normal cells, they can stimulate these same normal cells.

In all 25 control instances where normal cells were tested in MLC reactions—both responding and stimulating—were strongly positive. Of interest were several experiments performed using leukocytes from patients with rheumatic heart disease studied in conjunction with normal control cells. No impairment of response or stimulation was noted among these patients’ leukocytes with inactive rheumatic heart disease.

Testing for lymphocytotoxic antibodies and for blocking of normal MLCs with plasma from patients with ARF was negative. No evidence could be obtained using those technics for any MLC-blocking substance in the plasma of such patients.

Discussion

The results reported above appear to indicate some sort of cellular deficiency or anomalous behavior among lymphocytes from patients with acute rheumatic fever when studied in the one-way mixed-leukocyte culture. Apparent defects in the lymphocyte in the rheumatic state response have been previously demonstrated by several groups of investigators. In 1964 Hirschhorn et al. noted a diminished in vitro reactivity to streptolysin S among subjects with RF. Streptolysin S itself is not mitogenic. The mitogen present in streptolysin S preparations is a contaminant which can be separated from streptolysin S by chromatography and appears to be clearly distinct from streptolysin S. More recently Francis and Oppenheim recorded hyporesponsiveness in peripheral-leukocyte cultures stimulated by pathogenic strains of group A streptococci when patients with RF were compared to normal controls. Our findings of frequent lack of cross-stimulation in mixed-leukocyte cultures using lymphocytes from patients with active rheumatic fever are in accord with these previous documentations of lymphocyte hyporeactivity somehow associated with the acute rheumatic state. Similar observations have been previously recorded from this laboratory for subjects with active rheumatoid arthritis. This latter observation has very recently been confirmed and extended by Hedberg et al. in Sweden.

The apparent lymphocyte reactivity decrement documented in the MLC studies reported here may provide an important clue as to one of the basic immunologic derangements present in the acute rheumatic state. Of considerable interest in this regard are the recent reports by Malakian and Schwab documenting an immunosuppressant factor derived from group A streptococci. If such material is transferred to the susceptible host during incubation or generation of acute rheumatic fever, it might explain the hyporesponsiveness of lymphocytes recorded in the present study. Somewhat against this general notion is the impression that the group A streptococcal immunosuppressant studied by Malakian and Schwab is currently felt to exert its primary effect on the B-cell or bursa-derived component population of lymphocytes. The stimulation normally recorded in MLC using two unrelated histoincompatible donors is generally felt to represent a response of the T-cell or thymic-derived lymphocyte population.

It is conceivable that the MLC lymphocyte hyporeactivity recorded here among patients with acute rheumatic fever may be a nonspecific accompaniment of the disease itself, perhaps no more specific than elevation of the sedimentation rate, acute phase reactions, or fever. More data are needed on clinical correlations between lymphocyte hyporeactivity and the temporal course of disease or
carditis in individual patients before this question can be settled.

Recently Ceppellini et al.\textsuperscript{18} have shown that inhibition of human MLC activation can occur in the presence of serum with anti-HL-A activity, which may cover up or block antigens on stimulator or responder lymphocytes and thus abrogate cell stimulation. Careful testing of our sera from patients with acute rheumatic fever failed to demonstrate cytotoxic or blocking antibodies of this type. It is possible of course that certain HL-A antigens cross react with streptococcal cell-wall products. Some precedent for such an idea may be derived from the reports of Hirata et al.\textsuperscript{19} indicating cross reactivity between M-1 streptococcal protein and several human HL-A antigens. If during the course of acute rheumatic fever, patients developed antibodies to M protein or other streptococcal antigens that cross reacted with human cell-surface antigens, particularly materials present on the surface of lymphocytes, such antibodies could conceivably block or interfere with cross stimulation in the mixed-leukocyte culture. However, no evidence for such blocking antibodies was obtained in the present study. On the other hand, in acute rheumatic fever, cells sensitized to streptococcal antigens which cross react with human HL-A or surface lymphocyte structures might not recognize lymphocytes from other patients similarly sensitized to the same streptococcal antigens.

Finally, the present observations of apparent decreased lymphocyte reactivity in patients with acute rheumatic fever are difficult to reconcile with the role of the lymphocyte itself in the disease state. It is now abundantly clear that immune or sensitized lymphocytes are capable of mediating tissue damage through the production of various lymphokines such as migration inhibition factor MIF\textsuperscript{27} or cytotoxic factors.\textsuperscript{20, 21} Recently Perlmann\textsuperscript{22} has reviewed the various ways in which tissue damage can be mediated by circulating lymphocytes. When one examines the acute or subacute lesions of rheumatic carditis, it is indeed striking to note the prominence of various forms of lymphocytes in the tissues. It seems that it is now high time for investigators interested in basic mechanisms of tissue damage in rheumatic fever to pay more attention to the ubiquitous mediator of cellular immunity, namely the circulating lymphocytes. The present study serves merely to point out that, by the criterion of MLC, circulating lymphocytes in ARF appear to be abnormal. The exact link if any between lymphocyte abnormalities and the tissue lesions of rheumatic fever remains to be elucidated.

\textbf{References}


4. Friedman I, Lauffer A, Ron N, Davies AM: Experimental myocarditis: \textit{In vitro} and \textit{in vivo} studies of lymphocytes sensitized to heart extracts and group A Streptococci. Immunology 20: 225, 1971


\textit{Circulation, Volume XLVI, October 1972}
Decreased Reactivity of Lymphocytes in Mixed-Leukocyte Culture from Patients with Rheumatic Fever
RICHARD D. LUEKER and RALPH C. WILLIAMS, JR.

Circulation. 1972;46:655-660
doi: 10.1161/01.CIR.46.4.655
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1972 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/46/4/655

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/