Abnormal Autologous Mixed Lymphocyte Reaction in Autoimmune Thrombocytopenic Purpura

By Morton Zinberg, Tova Francus, Marc E. Weksler, Gregory W. Siskind, and Simon Karpatkin

The autologous mixed lymphocyte reaction (auto-MLR) measures the ability of non-T cells to stimulate autologous T cells to proliferate in tissue culture. The auto-MLR was studied in 11 patients with autoimmune thrombocytopenic purpura (ATP). Seven patients had decreased auto-MLR, which averaged $4440 \pm 3364$ cpm (SEM) compared to $15,360 \pm 6905$ cpm for simultaneously studied controls. The average of the ratios of cpm incorporated by patients/cpm incorporated by control subjects was $0.20 \pm 0.06$ ($p < 0.01$). Serum from all 7 patients with low auto-MLR decreased the auto-MLR of normal subjects by an average of $56 \% \pm 8.5 \%$ ($p < 0.001$). Preliminary results indicate that the inhibitory effect was mediated by a component of the IgG immunoglobulin fraction of serum. Sera from normal persons and from ATP patients with normal or high auto-MLR did not affect the auto-MLR of normal subjects. It was further shown that non-T cells from 3 of 5 patients with decreased auto-MLR failed to stimulate allogeneic T cells normally. It is concluded that many patients with ATP have decreased auto-MLR apparently due to the presence of a serum blocking factor and, perhaps, a defective stimulatory capacity of non-T-cells.

Little is known concerning the regulation of autoantibody production in autoimmune thrombocytopenic purpura (ATP). The autologous mixed lymphocyte reaction (auto-MLR) is a reaction in which T lymphocytes are stimulated in culture by autologous non-T-cells to proliferate. This phenomenon has the typical characteristics of immunologic reactions: memory and specificity. It has been proposed that the autologous-MLR reflects a normal in vivo interaction between T and non-T-cells that may be involved in the regulation of immune function.

Suppressor cells are generated during an in vitro auto-MLR. Patients with systemic lupus erythematosus (SLE) and NZB mice have both abnormal suppressor cell activity and impaired auto-MLR.

In a previous study we found that lymphocytes from patients with ATP were impaired in their responses to phytohemagglutinin and to concanavalin-A when whole blood lymphocyte preparations were tested. However, a normal response was observed with washed lymphocytes from the same patients. This suggested the presence of a plasma factor that could inhibit the response to lectins. The present report describes the auto-MLR of 11 patients with ATP who were not receiving any treatment when studied. Seven patients showed a reduced auto-MLR as compared with simultaneously studied controls. In the serum of these 7 patients, we detected a factor that decreased the auto-MLR.

MATERIALS AND METHODS

Patients Studied

The auto-MLR was investigated in 11 patients with ATP who were not receiving any therapy at the time they were studied. All had increased megakaryocytes in their bone marrow, detectable antiplatelet antibody, and/or had responded to steroid treatment or splenectomy in the past. Seven normal subjects served as controls.

Autologous MLR Cultures

The auto-MLR was performed as described previously. Briefly, human blood T lymphocytes that formed rosettes with sheep red blood cells (SRBC) were separated from non-T lymphocytes by Ficoll (Pharmacia, Piscataway, N.J.) Hypaque (Winthrop Laboratories, New York, N.Y.) centrifugation at 1400 rpm for 40 min at 4°C. The SRBC were lysed by resuspending the T cells in 0.83% ammonium chloride-0.17 M Tris buffer, pH 7.2. Seven percent of the T-lymphocyte preparation and 93%-98% of the non-T lymphocyte preparation reacted with an F(ab')2 fragment of rabbit anti-human Ig antibody that was labeled with fluorescein isothiocyanate. Responder T cells (10⁵) were mixed with an equal number of autologous or allogeneic irradiated (3000R) non-T-cells in 0.2 ml of culture medium. This contained RPMI 1640, 20% human AB serum, 9% penicillin-streptomycin, and 1% glutamine (Gibco, Grand Island, N.Y.). Cultures were incubated in triplicate in round-bottom Linbro microtiter plates (Flow Labs, McLean, Va.) for 6 days in an atmosphere of 5% CO₂-95% humidified air. Tritiated thymidine incorporation was measured during the last 24 hr of culture by adding 1 μCi of [³H]-methyl-thymidine (2 Ci/mM; Amershams/Searle Corp., Arlington Heights, Ill.) prior to harvesting the cells with a multiple well aspirating device. Incorporated radioactivity was assayed in a liquid scintillation counter. The average cpm for the triplicate cultures is reported.

Preparation of Serum Samples

Serum was prepared by allowing the blood to clot in glass test tubes at 37°C for 1 hr. The serum was separated from the clot by centrifugation and was heat-inactivated at 56°C for 30 min prior to storage at −20°C.

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RESULTS

Autologous MLR and Platelet Counts in ATP Patients

Eleven patients with ATP were studied (Table 1). The results of the auto-MLR of lymphocytes from 7 of the patients (numbers 1–7) were markedly lower than in simultaneously studied lymphocytes from normal subjects. For these 7 patients, the ratio of counts incorporated by patients’ lymphocytes to counts incorporated by control subjects’ lymphocytes averaged 0.20 ± 0.06 (SEM) (p < 0.01, Wilcoxon signed rank test) with a range of 0.01–0.61 (Table 1). While there is clearly a marked variation in the magnitude of the results of the auto-MLR of normal subjects, the degree of stimulation in the auto-MLR of 5 of these 7 patients is lower than the lowest degree observed among the 11 normal controls. Three of the 7 patients with impaired auto-MLR (numbers 5, 6, and 7) were restudied 5 mo later with similar results. Four patients (numbers 8–11) had auto-MLR that tended to be higher than those of simultaneously studied normal subjects, however, except for patient 8, they fell within the range of auto-MLR observed in normal subjects. Patient 11 was restudied 5 mo later and again gave a high auto-MLR response. No correlation was noted between disease severity and the auto-MLR.

Presence of a Serum Factor in Patients With ATP That Inhibits the Auto-MLR of Normal Subjects

Sera from all 11 ATP patients were tested for their capacity to block the auto-MLR of normal subjects (Table 2). Significant inhibitory activity (p < 0.01, paired Student t test) was detected in the serum of each of the 7 ATP patients whose auto-MLR was weaker than simultaneously studied normal subjects. When serum of these patients was present in the culture media at a concentration of 5% (v/v), there was an inhibition of the normal auto-MLR that averaged 56% (range 25%–89%). No inhibitory activity was detected in serum from the four patients (numbers 8–11) who did not have decreased auto-MLRs. In addition, serum from four normal subjects did not

<table>
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<tr>
<th>Table 1. The Auto-MLR in 11 Patients With ATP*</th>
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<tr>
<th>Table 2. Effect of ATP Patients’ Sera on Auto-MLR of Normal Subjects*</th>
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<td>Patient</td>
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*10^8 T cells were incubated with 10^8 autologous non-T-cells (irradiated with 3000 R) in a total volume of 0.2 ml of RPMI 1640, containing 20% AB serum, 1% penicillin-streptomycin, and 1% glutamine for 6 days at 37 °C in the presence of 5% CO_2. One microcurie of ^3H-thymidine was added during the last 24 hr of culture, and the lymphocytes then assayed for incorporation of thymidine into DNA.

†Splenectomized patients.
Table 3. Nature of the Suppressive Serum Factor in an ATP Patient With a Decreased Auto-MLR

<table>
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<tr>
<th>Additions to Culture</th>
<th>Auto-MLR (cpm x 10^4)</th>
<th>Percentage Suppression</th>
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<tr>
<td>20% normal AB serum</td>
<td>85.1</td>
<td>—</td>
</tr>
<tr>
<td>15% normal AB serum, 5% patient’s serum</td>
<td>26.9</td>
<td>68%</td>
</tr>
<tr>
<td>15% normal AB serum, 50 µg/ml patient’s IgG</td>
<td>26.1</td>
<td>69%</td>
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*Serum from patient number 3 was fractionated by 50% saturated ammonium sulfate precipitation and Sepharcl S300 gel filtration. The major peak of the retained volume gave a strong immunoprecipitation reaction with sheep anti-human IgG (and no reaction with anti-human IgM or IgA). Culture conditions were the same as described in the footnote to Table 1. Data are presented as average cpm for triplicate cultures.

significantly inhibit the auto-MLR of normal subjects when present at a concentration of 5% (average inhibition 7%; range 4%-15%). One patient’s serum (patient 3) was fractionated by 50% saturated ammonium sulfate precipitation and Sephacryl S300 gel filtration. The major peak of the retained volume gave a strong immunoprecipitation reaction in Oüchterlony plate with sheep anti-human IgG (heavy chain), but did not precipitate with sheep anti-human IgM or IgA. This IgG-enriched fraction inhibited the auto-MLR as strongly as did the original serum (Table 3). This suggests that the inhibitor might be an IgG immunoglobulin.

Non-T-Cells From Patients With Depressed Auto-MLR Do Not Stimulate Allogeneic T Cells Normally

Allogeneic MLC were examined using cells from normal subjects and from patients with ATP who had decreased auto-MLRs. Five patients with decreased auto-MLRs were studied. The T-cell population of these patients responded normally to allogeneic non-T-cells in each case (Table 4, columns 4 and 5). However, non-T-cells from three of the five patients studied did not stimulate allogeneic T cells normally (patients 2, 4, and 6 in Table 4, columns 2 and 3).

Patient 6 was studied on two occasions, 5 mo apart, with similar results.

DISCUSSION

The possibility that abnormalities in the auto-MLR might be related to the development of autoimmune disease was evaluated by studying the auto-MLR in ATP, a well defined autoimmune disorder. The autologous-MLR was studied in 11 patients with ATP who were not receiving any therapy at the time of the studies. Seven patients had markedly decreased auto-MLR as compared with simultaneously studied normal subjects. Five of these patients actually had auto-MLRs that were lower than the lowest observed in a normal subject. The depressed response was persistent, in that three patients who were retested after 5 mo exhibited the same abnormality as was observed on initial testing. The defect in auto-MLR does not appear to correlate with the clinical severity of the disease or with the degree of thrombocytopenia.

The older average age of the patient group (52 ± 4 versus 27 ± 1 for the control group) may have contributed to the lower auto-MLR of the patient group. However, this does not appear to be primarily responsible for the difference between both groups. Older subjects (>65 yr) have an approximate 1.6–2-fold lower auto-MLR than younger subjects (<30 yr). Since 5 of the 7 patients had a 12–84-fold lower auto-MLR than the control group, the age factor represents a small fraction of the decreased auto-MLR in our patient group. Furthermore, four patients with normal to increased auto-MLR had a comparable average age of 41 ± 8 yr.

It was found that serum from every one of the seven patients whose auto-MLR was weaker than that of simultaneously studied normal subjects contained a factor that depressed the auto-MLR of normal subjects. Preliminary data are consistent with the view that this blocking factor, which is not present in normal subjects or in ATP patients whose auto-MLR

Table 4. Evidence for a Defective Non-T Stimulator Cell in Some ATP Patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Patient Non-T-Cells With Control T Cells (cpm x 10^4)</th>
<th>Cell Populations Cultured Together</th>
<th>Control Non-T-Cells With Patient T Cells (cpm x 10^4)</th>
<th>Control “B” Non-T-Cells With Control “A” T Cells (cpm x 10^4)</th>
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<tbody>
<tr>
<td>2</td>
<td>1.4</td>
<td>27.5</td>
<td>46.3</td>
<td>31.7</td>
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<tr>
<td>4</td>
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<td>22.1</td>
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<td>29.5</td>
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<tr>
<td>7</td>
<td>14.9</td>
<td>18.3</td>
<td>67.6</td>
<td>18.3</td>
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</table>

*These allogeneic MLCs were performed with lymphocytes from ATP patients who had decreased auto-MLR. Culture conditions were as described in Table 1 except that stimulator non-T-cells and responder T cells were from different subjects as indicated. Control cultures were performed with mixtures of T and non-T-cells derived from two different control subjects (A and B). Data are expressed as average cpm for triplicate cultures.
is higher than that of simultaneously studied normal subjects, is an IgG immunoglobulin. Evidence for a serum factor that inhibits lymphocyte activation in patients with ATP has also been reported by Quaglia and Karpatkin, who found that lectin-induced blastogenesis was defective with unwashed lymphocytes from patients with ATP, but was normal when washed lymphocytes were employed. Thus, it seems likely that one of the causes of the decreased auto-MLR in patients with ATP is the presence of an inhibitory factor(s) in the IgG fraction of their serum.

Five of the ATP patients with decreased auto-MLR were evaluated for the capacity of their non-T-cells to stimulate an allogeneic MLR. Three of these five patients had defective stimulatory capacity. Such a defect might be responsible for their decreased auto-MLR. However, it is not certain that the same subset of non-T-cells is involved in stimulating both the allogeneic and autologous MLRs. Therefore, one cannot definitively conclude from the defective stimulatory capacity in the allogeneic MLR that there is also defective stimulatory capacity in the auto-MLR.

The relationship between the abnormal auto-MLR and the autoimmune process deserves some discussion. Depressed auto-MLRs have also been observed in patients with SLE, Hodgkin's disease, and in aged subjects. Both SLE and aging are associated with increased autoantibody production. It has been shown that suppressor cell activity is generated during an auto-MLR. It has been suggested that in vivo, the auto-MLR functions to generate suppressor activity that acts to down-regulate the immune response and to protect against autoimmunity. In the absence of an auto-MLR, one of the mechanisms for protection against autoimmunity fails to operate and autoimmune disease can result. However, this view may represent an oversimplification since: (A) there is an increased or normal auto-MLR in one-third of the patients with ATP, and (B) aged subjects often have increased nonspecific suppressor cell activity despite a decreased auto-MLR and an increase in autoantibody production. An alternative view is that the auto-MLR is actually operating maximally in vivo in response to the ongoing autoimmune process. As a consequence, cells normally responsible for stimulating or responding in the auto-MLR are depleted (or sequestered or consumed) from the peripheral blood by an unknown mechanism(s). In vitro testing would thereby yield a depressed response. According to this interpretation, the abnormality of the auto-MLR is not the primary defect leading to autoimmune disease but is rather a secondary consequence of the disease state. At present it is not possible to distinguish between these alternative hypotheses.

REFERENCES

Abnormal autologous mixed lymphocyte reaction in autoimmune thrombocytopenic purpura

M Zinberg, T Francus, ME Weksler, GW Siskind and S Karpatkin