Aplastic Anemia: Failure of Patient Leukocytes to Stimulate Allogeneic Cells in Mixed Leukocyte Culture

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One-way mixed leukocyte cultures were used to study the ability of cells from 53 patients with aplastic anemia and 65 of their HLA-identical siblings to stimulate and to respond to cells from unrelated individuals. The capacity of leukocytes from 28 of 53 patients to stimulate was diminished or absent, whereas their ability to respond remained comparable to that of cells from their HLA-identical siblings. Leukocytes from the group of patients with the lowest peripheral lymphocyte count were least stimulatory. The results indicate that in acquired aplastic anemia there exists a dissociation between stimulation and response in mixed culture.

APLASTIC ANEMIA is a hematologic disorder primarily associated with the disappearance and continued absence of marrow stem cells. Transplantation of healthy marrow from a sibling matched at the major histocompatibility complex (MHC) represents one form of therapy for this condition. Potential marrow donors and recipients are therefore tested by HLA serotyping, and those pairs found to be identical for HLA antigens are then tested by the mixed leukocyte culture (MLC) test. The MLC test measures the proliferative response of lymphocytes to cell membrane differences governed by a locus within the MHC that is presumed to be important in determining the outcome of a tissue transplant. Siblings who are genotypically identical for HLA antigens do not stimulate each other in MLC, except in the rare instance of a crossover within the MHC.

In 1973, Twomey et al. reported that leukocytes from four of six patients with aplastic anemia failed to stimulate cells from unrelated (allogeneic) individuals in a macrophage-poor mixed culture, although they did stimulate in a macrophage-enriched culture. Similarly, Sasportes et al. reported a patient with marrow aplasia whose lymphocytes did not stimulate but did respond to unrelated lymphocytes. To determine the frequency of this dissociation between stimulation and response, we analyzed MLC results—obtained in the course of tissue typing for marrow transplantation—on 53 patients with aplastic anemia and 65 of their HLA-identical siblings. Theoretically, cells from a patient and his MHC-identical sibling should represent the same degree of histoincompatibility to cells from any given unrelated individual and they should, there-
fore, stimulate the latter to a similar degree. Any significant difference in the ability of patient cells and sibling cells to stimulate would be attributable to aplastic anemia per se.

**MATERIALS AND METHODS**

**Patients and Siblings**

The 53 patients with aplastic anemia had severely depressed marrow function, with at least two of the following characteristics in the peripheral blood: platelet count <20,000/cu mm, granulocyte count <500/cu mm, and reticulocyte count (corrected) <1.0% with Hb <10 g/100 ml (nontransfused). The peripheral lymphocyte counts of the patients ranged from 200 to 4000/cu mm (median 1450/cu mm) at the time of testing. The aplastic anemia was associated with drugs or chemicals in nine patients, hepatitis in six, acquired paroxysmal nocturnal hemoglobinuria in two, Fanconi’s syndrome in two, and no known etiology in 34. Clinical details of 24 of the patients have been described previously. Most patients had received courses of steroid and androgen therapy. In most instances, therapy was stopped at least 24 hr prior to MLC testing. All but two patients had received transfusions of red cells or platelets. HLA serotyping was performed using cytotoxic antisera according to the method of Mittal et al. Fifty-three patients were found to be genotypically HLA identical with one or more of their siblings and were then studied in MLC.

**Mixed Leukocyte Cultures**

Cells from each patient-sibling pair were tested in MLC concurrently with those of one unrelated individual. Thus, patient-donor compatibility was assessed, and, at the same time, the ability of patient leukocytes and normal sibling leukocytes to stimulate and respond to the same allogeneic cell was compared.

Mixed cultures were carried out using the microplate method of Hartzman et al. with modifications. Mononuclear leukocyte suspensions were prepared from freshly drawn heparinized peripheral blood by centrifugation and flotation on Ficoll-Isopaque. This procedure yielded cell suspensions containing 80%-95% lymphocytes and 5%-15% monocytes, with less than 5% granulocytes for normal individuals, and suspensions of 85%-99% lymphocytes and 0%-12% monocytes for patients. The cells were washed three times in Waymouth MB 752/1 tissue culture medium (Gibco, Grand Island, N.Y.) and resuspended in medium. The culture medium was supplemented with 20% fresh human plasma from unrelated healthy individuals and with antibiotics. Thus, autologous patient plasma was always replaced with normal human plasma to eliminate any serum factors which might diminish or enhance an in vitro response.

Cultures were prepared in Falcon microtiter plates by mixing $1.5 \times 10^5$ responding lymphocytes with $4 \times 10^5$ stimulating leukocytes in a total volume of 0.2 ml. In some experiments varying doses of stimulating cells were used. To prepare stimulating cells for a one-way test, mitosis was inhibited by irradiation with 2500 R from a cesium source at a rate of 450 R/min. To insure that stimulating cells were indeed inactivated, every experiment included a control which demonstrated that irradiated cells from the patient did not respond to irradiated leukocytes from unrelated individuals. Triplicate cultures were incubated at 37°C for 6 days in a humidified atmosphere of 5% CO₂ in air. Three hours before harvesting, each culture was labeled with 2 μCi of H-thymidine (specific activity 6.7 Ci/mM; New England Nuclear, Boston, Mass.). Cultures were harvested by aspiration onto glass fiber filters using a multiple sample harvest device. Acid-insoluble material was precipitated and washed with 5% trichloroacetic acid, and the individual filters transferred to 5-ml counting vials. Three milliliters of scintillation fluid (BBOT in toluene, 4 g/liter; Packard Instrument Company) were added and the radioactivity assayed in a liquid scintillation spectrometer. Results are expressed in counts per minute (cpm) as the mean of triplicate cultures. A stimulation index (SI) was defined as mean cpm in the test mixture divided by mean cpm in the autologous control mixture. A log transformation of SI values was made and an F test was used to determine significance of correlation between groups of data.
RESULTS

The ability of patient leukocytes to stimulate allogeneic lymphocytes was compared with that of normal sibling cells to stimulate the same allogeneic cells. Figure 1 presents the results in the form of the SI and shows that the ability of patient cells to stimulate unrelated cells was markedly reduced. The median SI in response to patient leukocytes, shown in Fig. 1A, was only 9, with a range of 1–381. The corresponding cpm, not graphed, were a median of 7809 with a range of 95–137,000. By contrast, cells from siblings HLA identical with the patients stimulated the same allogeneic cells to a median SI of 62 and a range of 10–509 (Fig. 1B), with a median of 39,107 cpm and a range of 6312–267,679 cpm. In half of the patient-sibling pairs tested, patient leukocytes stimulated allogeneic cells with an SI of only 20° or less of the SI observed in response to the normal sibling cells. To rule out the possibility that similar differences in stimulation might be observed between cells of normal siblings, cells from 101 pairs of normal MHC-identical siblings were tested to compare their relative ability to stimulate allogeneic cells. The ratio between the SI in response to one member of a given pair and that in response to the other member ranged from 0.35 to 2.43, with a median ratio of 1.02. Thus, in no normal sibling pair was there a disparity of stimulation comparable to that observed in the majority of aplastic patient-sibling pairs.

To determine if hypostimulation was influenced by the concentration of stimulating cells, tests on 11 patients were performed with varying concentrations of stimulating cells. Leukocyte concentrations of $2 \times 10^9$, $6 \times 10^9$, and $10 \times 10^9$ per culture did not stimulate any more vigorously than did the standard dose of $4 \times 10^9$ cells.
The ability of patient lymphocytes to respond to allogeneic leukocytes was generally unimpaired and was comparable to that of their sibling cells. The median SI by patient cells responding to allogeneic cells was 136, with a range of 4-1794, reflecting a median cpm of 39,738 and range of 904-294,774. Cells from HLA-identical siblings responded to the same allogeneic leukocytes with a median SI of 83 (64,116 cpm) and a range of 8-439 (2995-225,936 cpm). The mild relative hyper-responsiveness of patient lymphocytes as represented by the SI reflects the mathematical influence of the somewhat lower autologous control cpm for patient cells (mean of 216 cpm) as compared to cells from the normal siblings (mean of 657 cpm).

The capacity of patient cells to stimulate was found to be related to the patient's absolute peripheral lymphocyte count. Patients were arbitrarily assigned to groups I, II, and III, according to the ability of their cells to stimulate allogeneic cells with SI of ≤3, 4-10, or >10, respectively. Figure 2 presents the absolute lymphocyte counts of patients in groups I, II, and III. Although leukocytes from some patients with a high peripheral lymphocyte count were unable to stimulate allogeneic cells and cells from some patients with low peripheral lymphocyte counts stimulated normally, there was a positive correlation among the groups between low absolute lymphocyte count and failure to stimulate in mixed culture (p <0.025). The concentrations of cells in the mixed cultures were, of course, adjusted so that the stimulating mixture always contained the same number of mononuclear leukocytes, irrespective of a patient's absolute lymphocyte count.

The patients' cells used for stimulation contained 0%-12% monocytes, with a median of 2%. There was no correlation between presence or absence or number of monocytes in the patients' stimulating mixtures and the ability to stimulate allogeneic cells (p >0.05).
There was no association between hypostimulation and any known etiology of aplastic anemia.

Table I presents the MLC data obtained from a representative patient in group I. The data illustrate the inability of patient cells to stimulate cells from an unrelated individual and the relatively normal response by patient cells to unrelated cells.

**DISCUSSION**

Cells from five patients with severe aplastic anemia have been reported to be capable of responding to allogeneic cells but unable to stimulate allogeneic lymphocytes in mixed culture unless macrophage-rich suspensions were used.4,5 Our results on 53 patients demonstrate the high incidence of such dissociation in aplastic anemia and, by concurrent tests on HLA-identical siblings, rule out the possibility that the lack of stimulation reflected a fortuitous histocompatibility between the nonstimulating patient cells and the allogeneic cells. Cells from 28 of 53 patients either failed to stimulate allogeneic cells or stimulated them far less vigorously than did cells from the patients' HLA-identical siblings.

Several possible explanations exist for the decreased ability of cells from patients with marrow failure to stimulate in mixed culture: (1) aplastic anemia may be associated with the loss of a population of cells specifically responsible for stimulation in MLC; (2) MLC-determinant sites at the cell surface may somehow be lost, altered, or blocked; (3) therapy for aplastic anemia may affect stimulation in MLC; and (4) conditions of the assay may not have been optimal for detection of stimulation by aplastic anemia patient cells.

The cell population responsible for stimulating in MLC has not been conclusively identified. Stimulation has been considered to be predominantly a function of B lymphocytes,10,11 T lymphocytes,12 or both.13,14 In addition, macrophages are known to be essential for MLC activation.15 Morley et al.16 reported depletion of B cells from the peripheral blood of nine patients with chronic hypoplastic marrow failure, but the ability of cells from these patients to stimulate in MLC is unknown. Our observation that cells from aplastic patients with low peripheral lymphocyte counts tend to be poor stimulators is also consistent with the possibility of stimulator-cell depletion. One can speculate that this depletion is of the B cell line, representing the loss of a population of short-lived cells normally produced in the bone marrow and responsible for the ability to stimulate in MLC. Long-lived T cells, on the other hand,
might remain in near-normal numbers in patients with aplastic anemia, and this might explain the ability of cells from our patients to respond relatively normally in MLC. This normal MLC responsiveness—reflecting T cell function—and Morley’s observation both support the concept of a B cell defect in such patients. The effect, if any, of monocyte depletion would not be apparent in our test system, since our cultures always contained monocytes, either in the responding or stimulating cell suspensions. Moreover, cells from seven patients who had no detectable monocytes were still able to stimulate normally.

Since stimulation and response in MLC may be controlled by separate but closely linked genetic loci, a decrease or loss of ability to stimulate without loss of ability to respond might result from blocking or alteration of the MLC-activating site at the cell surface. Greenberg et al. and Gatti et al., for example, have shown that some sera can block selectively the ability to stimulate in MLC while leaving intact the ability to respond. However, a serum blocking or inhibitory factor is an unlikely explanation for our results since patient serum was not used, and patient cells were extensively washed and the cultures supplemented with plasma from healthy unrelated individuals. This procedure, however, does not totally rule out this explanation. Alteration or loss of cell surface antigens must also be considered in view of the report that lymphocytes cultured at 22°C for more than 4 days lose their ability to stimulate allogeneic lymphocytes, yet retain their ability to respond. Although analogous loss or shedding of stimulating antigens from the cell surface as an explanation for our results cannot be ruled out, cells from all patients could be typed with cytotoxic antisera, and in no instance were patient lymphocytes found to lack HLA antigens expected from family segregation analyses.

Most of the patients studied had received androgens and/or steroids at various times before testing. Although it is possible that therapy may alter the ability of cells to stimulate in MLC, no supportive data are available. Moreover, 3 of the 17 patients whose cells failed to stimulate in MLC had not received any androgens or steroids.

It is also possible that the MLC assay itself affects the stimulating ability of cells from aplastic patients. For example, the cells used as stimulator cells may be more sensitive to the radiation used to block DNA synthesis than are normal leukocytes whose ability to stimulate is known to decrease with increasing levels of ionizing irradiation.

Another possibility to be considered is that the assay conditions were not optimal for stimulation by patient cells. The kinetics of the MLC reaction, for example, may be different for such cells in that the peak of DNA synthesis in responding cultures may be earlier or later than day 6. Moreover, the optimal number of stimulating cells may be different from the standard dose used. However, increasing or decreasing the number of patient cells used as stimulator cells did not alter the results in the nonstimulatory cultures. These results also make it less likely that antigens responsible for stimulation are present in reduced or subthreshold amounts on the patient cells. The last possible explanation for hypostimulation which requires further investigation is one mentioned by Sasportes et al., namely, that the stimulator patient cells somehow (without dividing) are cytotoxic to the viable responding allogeneic cells. This possibility has not been investigated.
The problem of hypostimulation by cells from patients with aplastic anemia must be recognized as being of practical importance for histocompatibility typing. Until the explanation for hypostimulation is determined and a method for rendering such patient cells capable of stimulating is devised, the possibility of obtaining false negative data in MLC studies prior to marrow transplantation must be appreciated.

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Aplastic anemia: failure of patient leukocytes to stimulate allogeneic cells in mixed leukocyte culture

EM Mickelson, A Fefer and ED Thomas