Demonstration of clonable alloreactive host T cells in a primate model for bone marrow transplantation
(total body irradiation/graft rejection/histocompatibility)

YAIR REISNER*, ISAAC BEN-BASSAT†, DAN DOUER‡, AMI KAPLOON‡, ELI SCHWARTZ*, AND BRACHA RAMOT‡

*Department of Biophysics, The Weizmann Institute of Science, Rehovot, Israel; †Institute of Hematology, The Chaim Sheba Medical Center, Tel-Hashomer, Sackler School of Medicine, Tel-Aviv University, Israel; and ‡The Biological Institute, Nes-Ziona, Israel

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ABSTRACT The phenomenon of marrow rejection following supralethal radiochemotherapy was explained in the past mainly by non-T-cell mechanisms known to be resistant to high-dose irradiation. In the present study a low but significant number of radiochemoresistant-clonable T cells was found in the peripheral blood and spleen of Rhesus monkeys following the cytoreductive protocol used for treatment of leukemia patients prior to bone marrow transplantation. More than 95% of the clonable cells are concentrated in the spleen 5 days after transplant. The cells possess immune memory as demonstrated by the generation of alloreactive-specific cytotoxicity. The present findings suggest that host-versus-graft activity may be mediated by alloreactive T cells. It is hoped that elimination of such cells prior to bone marrow transplantation will increase the engraftment rate of HLA-nondetected marrow in leukemia patients.

The application of bone marrow transplantation for the treatment of malignant and nonmalignant hematological disorders is largely limited to 30–40% of these patients who have an HLA-matched sibling. Transplants of marrow from haploidentical or fully mismatched donors regularly cause lethal graft-versus-host disease (1, 2). The successful use of T-cell-depleted haploidentical parental marrow for the transplantation of children with severe combined immune deficiency has shown that graft-versus-host disease can be prevented and should, therefore, no longer present a major obstacle in allogeneic transplantations (3–7). However, other problems were encountered when this approach was used for the treatment of patients with acute leukemia. O'Reilly et al. (8) have reported their experience with 42 leukemia patients, of whom 20 received transplants from HLA-nondetected donors. Of the 22 patients who received transplants from HLA-identical donors, 2 rejected their grafts, and 20 achieved a durable engraftment. In contrast, only 8 of the patients receiving HLA-nondetected grafts achieved durable engraftment, 2 required booster grafts, and 10 patients either failed to engraft or experienced graft rejection. The significant difference in rejection rate between recipients of HLA-identical and HLA-nondetected T-cell-depleted marrow strongly suggests that a population of radiochemoresistant cells with an HLA-restricted immune function persists in many leukemic patients despite the heavy conditioning prior to bone marrow transplantation. While the elucidation of this radiochemoresistant immunity is important, in particular for the design of new immunosuppressive regimens, this task is hampered by the extremely low counts of lymphocytes in the peripheral blood of irradiated patients. We, therefore, investigated whether clonable T lymphocytes can be detected in the peripheral blood or lymphatic tissues of primates after total body irradiation and chemotherapy treatment similar to that employed in the conditioning of leukemia patients prior to bone marrow transplantation (9).

MATERIALS AND METHODS

Animals. Colony-born Rhesus monkeys aged 3–5 years were used. The five males weighed 8–11 kg each and the two females weighed 3 and 5 kg.

Conditioning Regimen. The radiation and chemotherapy regimen was adapted from that developed for the conditioning of leukemia patients prior to bone marrow transplantation (9). Briefly, hyperfractionated total body irradiation, comprising 11 fractions of 120 cGy each delivered from a cobalt-60 source at a rate of 15 cGy/min three times per day on days –7 to –4, was given to the sedated monkeys without lung shielding. On days –3 and –2, cyclophosphamide (60 mg/kg) was administered intravenously. Four of the monkeys were sacrificed on day 0 (designated as the day when bone marrow transplantation would have been performed), and three monkeys were supported for 5 additional days and then sacrificed. The monkeys were kept in reverse isolation, fed sterile food, and given 100,000 units of nystatin and 40 mg of gentamycin sulfate orally three times daily. All procedures were performed after sedation with ketamine (10 mg/kg).

Cell Preparation. Normal saline solution was perfused through the great vessels, and 600–1000 ml of the perfusate was collected. The spleen and accessible lymph nodes were dissected, and the cells were suspended in Mg2+/Ca2+-free phosphate-buffered saline, pH 7.4. The mononuclear cells were isolated by centrifugation on a Ficoll/Hypaque gradient. The E-rosette assay was performed as described (10). Isolation of T lymphocytes from the peripheral blood mononuclear cells was carried out by the E-rosetting technique using sheep erythrocytes as described (3).

Limit Dilution Analysis for Clonable T Cells. The method of Chen et al. (11) was slightly modified. Briefly, cultures (200 μl) were set up in flat-bottomed microtiter trays. The medium was RPMI 10% (vol/vol) fetal calf serum. The stimuli were phytohemagglutinin (0.9 μg/ml, Wellcome) and a serum-free conditioned medium (20%) from the Gibbon T-cell line MLA144 (12). This line releases interleukin 2 constitutively without detectable levels of other lymphokines and cytokines (13). Each culture contained 1 × 104 irradiated (30 Gy) allogeneic peripheral blood lymphocytes as feeder cells. The responding cell concentration ranged from 0 to 20,000 cells per well for the treated animals and from 0 to 10,000 cells per well for the untreated animals. Between 12–48 culture wells were set up for each cell dose. The culture time was 8 days at 37°C in a humid, 5% CO2/95% air atmosphere incubator. The cultures were pulsed with 0.5 μCi (1 Ci = 37 GBq) of [3H]thymidine 12 hr before harvest, and the incorporation into DNA was determined by scintillation counting. Test wells were considered positive when [3H]thymidine incorporation exceeded mean background counts by at least
Distribution and Recovery of Mononuclear Cells. Only a minute number of mononuclear cells could be recovered from the treated monkeys. The lymph nodes were severely atrophic and viable cells could not be found in them, neither on day 0 nor on day 5. Viable mononuclear cells were present only in the spleen and in the peripheral blood. The mean numbers of mononuclear cells that could be recovered from these sources on day 0 and on day 5 are shown in Table 1. As can be seen, most of the mononuclear cells on day 0 were found in the peripheral blood, while on day 5 they were concentrated in the spleen. Using the E-rosette assay with sheep erythrocytes, a significant proportion of the mononuclear cells were found to be T cells. Again, almost all of these T cells were concentrated at day 5 in the spleen (Table 1).

Limit Dilution Analysis of Clonable T Cells. A typical limit dilution analysis of clonable T cells from an irradiated and a control (untreated) monkey is shown in Fig. 1. The frequency of clonable cells, calculated from the slopes of the straight lines, was $2 \times 10^{-4}$ for the irradiated monkey and $15 \times 10^{-4}$ for the untreated animal. Clonable T cells were found by this assay in six of the seven monkeys tested; there was no difference in the cloning efficiency on day 0 compared to day 5, and, therefore, the results were combined. The mean cloning efficiency of T cells in the blood of the irradiated monkeys was $1.1 \times 10^{-4}$ compared to $9.4 \times 10^{-4}$ in seven control untreated animals. The ratio between the cloning efficiency of untreated and treated animals in each separate experiment ranged between 7.5 and 13.5. On the average, 500 ml of blood was withdrawn from irradiated monkeys. The average total number of mononuclear cells collected was $6.8 \times 10^{9}$ at day 0 and $0.95 \times 10^{6}$ at day 5, compared to $5-7 \times 10^{9}$ from untreated animals. This 2-logarithm reduction in the total number of mononuclear cells in the peripheral blood of treated animals, together with the reduction in cloning efficiency, results in a total 3-logarithm depletion of peripheral blood clonable T cells in the treated monkeys. In two experiments, E-rosette positive cells were isolated from the blood of the conditioned monkeys and then subjected to the limit dilution analysis. As can be seen in Fig. 2, the cloning efficiency was not reduced in this purified T-cell fraction.

Alloreactive Cytotoxic Precursors in Spleen of Conditioned Monkey. Splenic cells from conditioned monkey A obtained at day 5 were cocultured with feeder cells from normal monkey B, and 7 days later were tested for their capacity to

Table 1. Total number of mononuclear and E-rosette forming cells recovered from monkeys prepared for bone marrow transplantation

<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Day 5</th>
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</thead>
<tbody>
<tr>
<td>Peripheral blood</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mononuclear cells</td>
<td>6.8 ± 4.5</td>
<td>0.95 ± 0.9</td>
</tr>
<tr>
<td>E+ cells</td>
<td>1.6 ± 1.8</td>
<td>0.09 ± 0.09</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mononuclear cells</td>
<td>1.6 ± 1.8</td>
<td>27.4 ± 14.1</td>
</tr>
<tr>
<td>E+ cells</td>
<td>0.4 ± 0.7</td>
<td>7.5 ± 2.1</td>
</tr>
</tbody>
</table>

The values are presented as the (mean average ± SD) x 10^{-4}. The number of experiments for day 0 was 4 and for day 5 was 3. E+ cells, E-rosette forming cells.

three standard deviations. These data were then used in an equation based on the Poisson probability distribution, ln y = fx + In a, where y is the fraction of nonresponding cultures, x is the number of tested cells per culture, f is the frequency of clonable T cells among the tested cell population, and a is the y-axis intercept, equivalent to the background response in the absence of responder cells. The value of f was calculated from the slope of the line fitting the data points, using a least-squares linear-regression analysis.

Assay for Cytotoxic Precursors. Day 5 spleen mononuclear cells from the treated monkey A were sensitized in a 7-day culture against irradiated (30 Gy) peripheral blood mononuclear feeder cells from monkey B as described above, except that a macroculture containing $0.5 \times 10^{6}$ cells from monkey A and $1 \times 10^{6}$ cells from monkey B was set up in a 5-ml flask instead of the microtiter tray. At the end of the culture the capacity of the sensitized cells to lyse $^{51}$Cr-labeled phytohemagglutinin blasts from monkey B was compared to the cytotoxicity against $^{51}$Cr-labeled target cells from a third party, monkey C. The cytotoxicity index was calculated as follows.

$$\text{Cytotoxicity index} = \frac{a - b}{c - b} \times 100$$

where a is the cpm in the supernatant of the mixture of target and effector cells, b is the cpm in the supernatant of the target cells alone, and c is the cpm of the target cells after lysis with 5% Triton.
lyse target cells from monkey B. In two separate experiments, the cultured cells gave rise to cytotoxic cells specific for targets from monkey B and do not lyse target cells from a third party (monkey C) (Table 2).

**DISCUSSION**

The major obstacle to the use of HLA-nonidentical bone marrow transplants in leukemia patients following lethal radiochemotherapy is the high rate of graft rejection by the host. In murine models two major lymphocyte subpopulations have been implicated in bone marrow rejection. Natural killer cells have been extensively studied in this context over the past 10 years. In particular Cudkowicz and coworkers (14, 15) and others (16-18) have shown that these radioresistant cells are involved in the hybrid-resistance phenomenon. On the other hand radioresistant T cells were suggested as being involved in allogeneic inhibition of colony forming units in the spleen. Using lethally irradiated AKR mice treated in vivo with anti-Thy-1 antiserum prior to transplantation of BALB/c bone marrow cells, Von Melchner and Bartlett (19)

Table 2. Precursors of cytotoxic T lymphocytes in spleen of monkeys at day 5 after preparation for bone marrow transplantation

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Target, maximal</th>
<th>Target, spontaneous</th>
<th>Target plus culture (A x B)</th>
<th>Cytotoxic index, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B</td>
<td>11,894</td>
<td>2,857</td>
<td>7,237</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>6,036</td>
<td>1,830</td>
<td>4,184</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>2,228</td>
<td>261</td>
<td>2,489</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1,467</td>
<td>268</td>
<td>295</td>
</tr>
</tbody>
</table>

B, feeder cells. C, cells from 3rd party.

*Results were obtained at a 10:1 ratio of effector to target cells.

have shown that abrogation of the inhibition of colony forming units in the spleen could only be achieved by the anti-Thy-1.1 directed against host T cells and not by anti-Thy-1.2 specific for the donor cells. Further indication for the role of T cells was provided by the demonstration of HLA control in marrow rejection among leukemic patients (8), suggesting that this HLA-specific immunological barrier may be similar to graft-versus-host disease and is probably mediated by T cells rather than by less specific cells such as natural killer cells or macrophages, the functions of which are not restricted by the HLA loci (20).

The aim of the present study was to investigate whether clonable T lymphocytes can be detected in primates after total body irradiation and chemotherapy treatment similar to that used for the conditioning of leukemic patients prior to bone marrow transplantation. As expected, only minute numbers of mononuclear cells were obtained from the blood and spleens of these lethally treated monkeys. However, in every such experiment a significant number of T cells was found by the E-rosette assay with sheep erythrocytes. Interestingly, the majority of T cells at day 0 were found in the blood, whereas on day 5 they were concentrated in the spleen. Clearly, the presence of the T cells would be relevant to the phenomenon of graft rejection only if, after the heavy irradiation and chemotherapy, these cells are still capable of clonal expansion in response to the appropriate mitogenic stimulus. To answer this question, we adapted a limit dilution assay for clonable T cells, previously used to determine residual functional T cells in mouse thymus (11). By this method a single clone containing about 200 or more cells can generally be detected. Clonable cells were detected by this assay in six out of the seven treated monkeys at a frequency of one-tenth of that found in untreated animals. The limit dilution analysis in our study was dependent on the simultaneous presence of both phytohemagglutinin and exogenous interleukin 2, suggesting that the growth of non-T lymphocytes in this system is unlikely. Further evidence for the T-cell derivation of the clonable cells was provided by the finding that the cloning efficiency was not reduced following purification of T cells by E-rosetting of the peripheral blood mononuclear cells of the conditioned monkeys. Moreover, we found that the growing cells—unlike natural killer cells—possess immune memory as demonstrated by the generation of alloreactive specificity in the sensitization culture. Thus, our finding of clonable T cells in monkeys after treatment with a protocol commonly used to condition leukemic patients provides a definitive proof for the existence of these previously hypothesized T cells.

Eradication of the residual clonable T cells of the host by more effective immunosuppressive regimens may reduce the rejection rate of HLA-mismatched marrow transplants. This could be achieved by several approaches, including increasing the dose of total body irradiation (21), adding total nodal irradiation (22), intensifying chemotherapy, or using anti-T monoclonal antibodies. However, the finding that most of the T cells are concentrated at day 5 in the spleen of the conditioned monkeys suggests that this goal may be achieved by methods directed more specifically to this population.

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