Analysis at the Clonal Level of T-Cell Phenotype and Functions in Severe Aplastic Anemia Patients

By Maurizio Viale, Annalisa Merli, and Andrea Bacigalupo

The aim of this study was to analyze at the clonal level the phenotype and functions of T cells from patients with severe aplastic anemia (SAA). For this purpose we studied 175 T-cell clones obtained from peripheral blood (PB) and bone marrow (BM) of four SAA patients and 97 clones from two healthy controls. The percentage of CD8+ T-cell clones obtained from the patients' PB and BM was higher, but not significantly (P = .07 and P = .14, respectively), than that obtained in controls. A higher proportion of T-cell clones from SAA patients exhibited lectin-dependent cytolytic activity and especially natural killer-like activity when compared with controls (PB: P < .01, P < .05; BM: P < .05, P < .01, respectively). Lymphokine release was tested before and after mitogen stimulation. A number of patients' clones were able to release interferons (IFNs) spontaneously (PB: 28.6% v 0%, P < .05; BM: 28.6% v 0%, P < .10). After mitogen stimulation, patients' BM T-cell clones produced IFNs in greater proportions (90.9% v 46.7%, P < .01) and in greater quantities (PB: 25.5 arbitrary units [AU]/mL v 5.7 AU/mL, P < .03; BM: 26 AU/mL v 9.1 AU/mL, P = .011) as compared with controls. Tumor necrosis factor (TNF) activity was not found in supernatants of unprimed T-cell clones. After mitogen stimulation, PB T-cell microcultures produced TNFα in greater proportions (97.9% v 72.2%, P < .01) and, also in this case, in greater quantities (PB: 7.2 AU/mL v 1.5 AU/mL, P < .007; BM: 9.9 AU/mL v 1.5 AU/mL, P < .003) than controls. In conclusion, T-cell clones from SAA patients exhibit predominantly a CD8+ phenotype, a greater cytotoxic activity, and can be shown to produce greater quantities of suppressor lymphokines when compared with controls.

T HE IMMUNE SYSTEM is thought to play an important role in patients with severe aplastic anemia (SAA). This is suggested by a series of evidence, such as hematopoietic reconstitution after immunosuppressive therapy13 and improvement of in vitro colony formation after removal of T lymphocytes from the marrow of SAA patients.4 The latter observation prompted a number of studies on the role of accessory cells in the regulation of hematopoietic progenitors.3,5,6 Some investigators described a colony suppressor activity by exposing normal peripheral blood T cells to mitogens;7 the same suppressor activity is also spontaneously produced by T cells in aplastic patients.8 This suppressor activity has recently been shown to be, at least in part, mediated by lymphokines such as interferons (IFNs) or tumor necrosis factors (TNFs).9,10 All together, the information suggests that the immune system is either involved in the pathogenesis or in the maintenance of marrow failure in some patients with SAA.

The majority of in vitro studies, performed to phenotypically and functionally characterize T lymphocytes in aplastic patients, have been performed using bulk cultures, whereas no studies have been performed until now at the clonal level. For this purpose we have studied cytolytic properties and lymphokine production (IFNs, TNFs, interleukin-3 [IL-3]/granulocyte-macrophage colony-stimulating factor [GM-CSF]) of T-cell clones obtained from peripheral blood (PB) and bone marrow (BM) of SAA patients and normal individuals by limiting dilution analysis. This method is a useful system to obtain information about the functional repertoire of T cells.11

MATERIALS AND METHODS

Patients. Eight patients with SAA were studied for surface marker expression of T cells. There were three males and five females, and age ranged from 18 to 56 years. Four patients were transfusion dependent and four were transfusion independent, whereas none were infected at the time of study. All patients were treated with horse antilymphocyte globulin (ALG) (Merieux, Lyon, France), 637 lymphocytotoxic units/kg/d (50 to 150 mg of IgG/mL) on each of 5 consecutive days, followed by prednisolone 5 mg/kg/d. At present, seven patients are surviving and transfusion independent. One patient did not respond to ALG, was grafted, and died 1 year post bone marrow transplantation of late rejection, after discontinuation of cyclosporine A. Four patients (two males and two females, aged 18 to 48 years) were further studied at the clonal level.

Mononuclear cell (MNC) isolation. MNC were isolated from 20 to 25 mL of heparin-treated PB and 10 mL heparin-treated BM by density gradient centrifugation and washed twice with RPMI 1640 containing gentamycin (100 μg/mL), L-glutamine (2 mmol/L), nonessential amino acids (1% vol/vol), and 10% heat-inactivated fetal calf serum (complete medium).

Separation and cloning of T cells. MNC were resuspended in neuraminidase-treated sheep erythrocytes and purified by centrifugation over Ficoll-Hypaque gradient. E-rosetting cells (E+) were greater than 90% pure. Purified T cells were diluted in complete medium and seeded at 0.5 to 20 cells/well in round-bottomed microtiter plates containing 104 irradiated (50 Gy) allogeneic PB mononuclear feeder cells, 1% phytohemagglutinin (PHA), 100 U/mL recombinant (r) IL-2 (Glaxo Institute of Molecular Biology, Geneva, Switzerland), and 2% (vol/vol) supernatant (SN) derived from PHA-stimulated human spleen cells and deprived of PHA. Every 3 days microcultures were supplemented with 100 μL of rIL-2 (50 U/mL) and 2% SN containing complete medium. After 15 to 25 days of culture, microwells were scored microscopically for growth. Proliferating microcultures were operationally considered as clonal when less than 1/2 of the cells plated at a given cell number were scored as positive for proliferation, thus ensuring high probabilities of clonality. Clonal microcultures were then split into...
performed in the presence of 1% PHA using the controls' clones, [EA]). A 4-hour "Cr-release assay was
murine P815 cell line as target cells.

<table>
<thead>
<tr>
<th>CD3</th>
<th>CD4</th>
<th>CD6</th>
<th>CD56</th>
<th>CD25</th>
<th>DR</th>
<th>CD4/CD8</th>
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Table 1. Surface Marker Analysis of PB T Lymphocytes (top) and BM T Lymphocytes (bottom)

<table>
<thead>
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<th>CD3</th>
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<th>CD6</th>
<th>CD56</th>
<th>CD25</th>
<th>DR</th>
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<td>0.097</td>
<td>0.042</td>
<td>0.139</td>
<td>0.139</td>
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*The numbers represent the mean percentage of cells stained with the indicated MoAbs.

several microwells, expanded, and analyzed for surface marker expression and functional capabilities.

Surface marker analysis. Separated T cells from PB and BM of patients and healthy donors were analyzed for surface marker expression of CD3, CD4, CD8, CD25, CD56, and DR antigens; cloned cells were analyzed for the expression of CD3, CD4, and CD8 antigens. Cells were stained with OKT3, OKT4, Leu2a, Leu19, OKla, and anti-TAC monoclonal antibodies (MoAbs) followed by a fluorescein isothiocyanate-conjugated rabbit antirabbit- IgG. Positivity was determined by fluorescence microscope examination. OKT3, OKT4, and OKla MoAbs were purchased by Ortho Pharmaceuticals (Raritan, NJ); Leu2a and Leu19 from Becton Dickinson (Milano, Italy); and anti-TAC antibody (MAR 98 MoAb) was a kind gift of Dr A. Moretta (University of Genova, Genova, Italy).

Assays for cytolytic activities. Cytolytic activity was determined using a standard 4-hours 3Cr release assay. The cell lines used in these studies were: the human natural killer (NK)-sensitive K562 (NK-like activity), the murine P815 cell line in the presence of 1% PHA (lectin-dependent cellular cytotoxicity [LDCC]), allogeneic PHA lymphoblasts, and cryopreserved autologous bone marrow E- (NK-like activity), the murine P815 cell line in the presence of 1% PHA + 1 ng/mL 12-O-tetradecanoylphorbol 13-acetate (TPA), were added to 5 × 10^5 51Cr-labeled WEHI 164 sarcoma cells that had been previously pretreated for 3 hours with 1 μg/mL actinomycin D. After an 18-hour incubation period, supernatants were removed and 51Cr-release was assessed using a gamma counter. For TNF blocking experiments, each supernatant was incubated for 30 minutes at room temperature with appropriate dilutions of a rabbit antihuman TNFα polyclonal antiserum or a mouse anti human TNFβ MoAb (both provided by Genzyme, Boston, MA). Data were expressed in arbitrary units (AU) per milliliter, considering one unit as that able to induce the maximal cytolysis, obtained by a TNF-containing standard, in the assay.

IFN was assessed using human amnion cells (FLAM) in a 50% cytopathic effect reduction assay.14 FLAM indicator cells were seeded into microtiter flat-bottomed plates at 2 × 10^4 cells/well. After 24 hours, confluent cells were overlayed with twofold dilutions of supernatants and incubated for 48 hours. Then indicator cells were infected with vesicular stomatitis virus [100 plaque-forming units [PFU]/well] and incubated for an additional 24 hours. Culture plates were then washed, fixed with methanol, and stained with a 10% solution of Giemsa stain. IFN activity was expressed in AU per well obtained from the reciprocal of the highest dilution giving 50% reduction of the cytopathic effect in infected control cultures.

IL-3/GM-CSF was assayed using the IL-3/GM-CSF-dependent MO7 human cell line15 (kindly provided by L. Pegoraro, Osp. Molinette, Torino, Italy). Indicative cells were seeded at 2 × 10^4 cells/well in U-bottomed wells of microtiter trays in the presence of 15% dilution of each supernatant. All supernatants were tested in triplicate, whereas the final volume was 200 μL/well. After 72 hours, culture cells were pulsed with 1 μCi of tritiated thymidine [3H-TdR] for 6 hours and then harvested and counted in a beta counter. Data were expressed in a qualitative manner (positive or negative) considering as positive those supernatants with exceeding counts per minute (cpm) by at least 5 SD that of control.

**Figure 1.** Histograms represent the percentage of total PB- and BM-derived T-cell clones (left panel) and of CD4+ and CD8+ T-cell microcultures (right panel) with LDCC activity [patients' clones, [ ), controls' clones, [I]. A 4-hour 3Cr-release assay was performed in the presence of 1% PHA using the murine P815 cell line as target cells.
clones (M07 + complete medium). For IL-3/GM-CSF blocking experiments, each supernatant was incubated for 30 minutes at room temperature with opportune dilutions of specific mouse antihuman–GM-CSF and antihuman–IL-3 MoAbs (Genzyme) and then tested as described.

Statistical analysis. Chi-square, Student's t, Fisher's, and the Mann-Whitney tests were used for statistical analysis.

RESULTS

Surface marker analysis of PB and BM T cells and T-cell clones. The surface phenotype of T lymphocytes isolated from PB and BM of eight patients with SAA and four healthy donors was characterized using MoAbs directed against CD3, CD4, CD8, CD25, CD56, and DR antigens. As shown in Table 1, an increased percentage of CD56 and DR cells was found in patients' PB cells. Similarly, a higher number of CD8+ cells and a decreased percentage of CD4+ cells was observed with a significant reduction of the CD4/CD8 ratio. A similar phenotypical pattern was also found in the BM, in particular the CD4/CD8 ratio was significantly higher in controls than in patients, whereas the percentage of CD56+ cells was higher in patients. Taken together these results demonstrated that in PB and BM of most SAA patients the CD4/CD8 ratio is lowered due both to a deficiency of CD4+ cells and an increase of CD8+ cells. In addition, T cells carried T-cell activation markers such as DR, CD56, and, in some cases, CD25.

A total number of 152 PB and BM T-cell clones obtained in four SAA patients and 93 PB and BM T-cell clones obtained in two healthy donors were studied for their surface marker expression. All clones considered in this study were CD3+, whereas a higher percentage of CD8- microcultures was found in patients' PB- and BM-derived clones, although the difference did not reach statistical significance (Table 2). In particular, it is noteworthy that in both patients and controls the CD4/CD8 ratio found in freshly isolated T cells generally reflected the ratio between CD4+ and CD8- clones. This result suggested that clones considered in this study were representative of the starting T-cell population. Moreover, it should be noted that, in general, patients' T cells displayed a lower cloning efficiency than control T lymphocytes (PB: 16.5% v 50%; BM: 10% v 50%).

Cytolytic properties of T-cell clones. One hundred ninety-nine T-cell clones either derived from PB and BM of patients and healthy donors were tested for cytolytic activity in LDCC against the murine P815 tumor target cell line in the presence of 1% PHA. This assay allows the detection of any cytolytic T-cell clone irrespective of its specificity. Significantly higher percentages of cytolytic PB- and BM-derived T-cell clones were found in patients than in healthy donors (PB: 81.2% v 54.3%, P < .01; BM: 74% v 47%, P < .05). The majority of patient and control cytolytic clones were CD8+, although in patients higher percentages of LDCC+ microcultures were found either in CD4+ and CD8+ groups of clones (Fig 1).

Microcultures were further studied for their capability to kill the NK-sensitive K562 human cell line. Significantly higher percentages of PB- and BM-derived T-cell clones with NK-like activity were found in patients as compared with controls (PB: 33.8% v 11.8%, P < .05; BM: 37.5% v 3.2%, P < .01). Also in this case, stratifying for CD4+ and CD8+ groups of clones the percentage of NK-like cytolytic microcultures was significantly higher in patients than in controls (Fig 2).

T-cell clones were also tested for the cytolytic activity against autologous BM E- cells. Of 72 microcultures either derived from PB and BM of patients, five (6.9%) expressed a low but significant lytic activity, whereas 1 of 36 control clones (2.7%) was cytolytic in this assay. Although not statistically significant, this difference suggests the presence...
of T cells able to exert cytolytic functions against autologous BM T-depleted cells.

Finally, 26 clones obtained from PB or BM of two patients who had received blood transfusions and 58 derived from PB or BM of healthy donors were tested against a random panel of four to six allogeneic PHA lymphoblasts. It is worth noting that 30.8% of patients' clones were cytolytic in this assay at least against one target, whereas only 3.5% of control clones had the same cytolytic characteristics ($P < .001$). All of these cytolytic clones were CD8$^+$ and were able to release IFNs and TNFs after mitogen stimulation.

**Lymphokine production.** We further evaluated the ability of T-cell clones to release TNFs, IFNs, and IL-3/GM-CSF in response to mitogen stimulation. Microcultures were washed and stimulated for 24 hours with 1% PHA + 1 ng/mL TPA. Supernatants were then collected and tested in the different biologic assays as described in Materials and Methods.

We examined 114 patients' and controls' PB- and BM-derived T-cell clones for TNF production. A higher percentage of patient-derived PB T-cell clones was able to release TNFs after mitogen stimulation as compared with controls (97.9% $\times 72.2\%$, $P < .01$). In BM, no differences were found in the percentages of TNF-producing T-cell microcultures between patients and controls (97% $\times 100\%$, $P < .01$). In both PB, it is noteworthy that greater percentages of CD4$^+$ and CD8$^+$ clones were able to secrete TNFs in patients than in controls (CD8: 100% $\times 83.3\%$, $P < .05$; CD4: 100% $\times 81.8\%$, $P < .20$), although only for CD8$^+$ microcultures the difference was statistically significant. Moreover, in both PB- and BM-derived patient microcultures the mean amount of TNFs produced and evaluated as AU per milliliter was higher than that observed in control clones (PB: 7.2 $\times 1.5$, $P = .007$; BM: 9.9 $\times 1.5$, $P = .003$) (Fig 3). Experiments of TNF activity inhibition by the use of a mouse antihuman TNF$\beta$ MoAb or by a rabbit antihuman TNF$\alpha$ antiserum demonstrated that T-cell clones mainly released TNF$\alpha$ after mitogen stimulation (Fig 4).

One hundred sixteen PB- and BM-derived T-cell clones were tested for IFN production. Although no differences were found in the percentage of IFN-releasing microcultures from PB-derived patient and control clones (78.6% $\times 81.2\%$), a higher percentage of IFN-producing clones was found in patients' BM as compared with control BM (90% $\times 46.7\%$, $P < .01$). In addition, a greater percentage of CD8$^+$ microcultures was able to release IFNs in patients than in controls (Fig 5). It is worth noting that, unlike control clones, some patient PB- and BM-derived T-cell microcultures released IFNs spontaneously, without mitogen stimulation (PB: 28.6% $\times 0\%$, $P < .05$; BM: 28.6% $\times 0\%$, $P < .10$), and that after mitogen priming patients' clones produced higher numbers of AU per milliliter of IFNs than controls (PB: 25.5 $\times 5.7$, $P < .003$; BM: 26 $\times 9.1$, $P = .011$) (Fig 3).

Finally, although this difference was not statistically significant, there were less patients' PB- and BM-derived T-cell clones able to release IL-3/GM-CSF as compared with controls (PB: 84.8% $\times 100\%$, $P < .30$; BM: 77.3% $\times 100\%$, $P < .20$), as demonstrated by the biologic assay.
using the IL-3/GM-CSF–dependent M07 cell line. The decreased percentage of IL-3/GM-CSF–producing microcultures was in the CD8+ group of clones (CD8+: 79.5% v 100%, P < .10; CD4+: 96% v 100%, P < .70). Finally, the use of the M07 cell line, mouse antihuman-GM-CSF, and antihuman-IL-3 MoAbs demonstrated that GM-CSF was the lymphokine mainly produced by T-cell clones (Fig 6).

**DISCUSSION**

We analyzed at the clonal level T-cell phenotype and functions in patients with SAA. Although the clonal efficiency was not very high in patients as compared with controls, the CD4/CD8 ratio of CD4+ and CD8+ T-cell clones was generally similar to that observed in starting T-cell populations. This finding suggested that our microcultures were representative of starting T-cell populations. Increased CD8 positivity together with reduced CD4 positivity were seen in patients’ T-cell clones and in freshly isolated T lymphocytes. In addition, as described by others, most patients had increased percentages of lymphocytes expressing on their surface T-cell activation markers, such as DR, CD56, and CD25 (only in some patients). It is noteworthy that the CD56 antigen is present normally on virus-infected cells. For example, NK cells are accumulated such as DR, CD56, and CD25 (only in some patients). It is noteworthy that the CD56 antigen is present normally on virus-infected cells. For example, NK cells are accumulated.

The increased NK-like activity of T cells in patients capable of exerting LDCC activity is not in contrast with our findings, which relate only to T-cell–mediated cytolytic activity and therefore to T-cell repertoire. It is unlikely that ALG treatment, reported to enhance NK activity,24 would have influenced NK-like activity in our patients because it was given at a median interval of 10 months (range 4 months to 5 years) before the study.

Some patients had T-cell clones capable of cytolytic functions against autologous E+ BM cells. Although it is tempting to interpret this as an autoimmune reaction, we want to stress that some normal control clones had similar activity and, in addition, we cannot rule out alloimmunization from blood products. In keeping with the latter comment, two patients had a high frequency of alloreactive cytolytic T-lymphocyte precursors (CTLp) (Table 3). The possible clinical role of alloreactive CTLp is unknown, but one may consider their capacity to release IFNs and TNFα as relevant to the condition of marrow failure.

In this line, the overproduction of factors with suppressive effects on hematopoiesis has been extensively de-

**Table 3. Functional Characteristics of Patients’ T-Cell Clones:**

<table>
<thead>
<tr>
<th>T-Cell Clones</th>
<th>Patients v Controls</th>
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<tbody>
<tr>
<td></td>
<td>BM</td>
</tr>
<tr>
<td>LDCC activity</td>
<td>↑</td>
</tr>
<tr>
<td>NK-like activity</td>
<td>↑</td>
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<tr>
<td>Cytotoxicity against allogeneic PHA-lymphoblasts</td>
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<tr>
<td>% IFN-releasing clones:</td>
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<td>PHA + TPA stimulation</td>
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<tr>
<td>IFN-production/clone</td>
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<tr>
<td>% TNF-releasing clones:</td>
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<tr>
<td>PHA + TPA stimulation</td>
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<tr>
<td>TNF-production/clone</td>
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*Not significant, † increased and ‡ decreased values in patients as compared with controls, and analyzed separately for BM derived (BM) and PB derived (PB) T-cell clones.
scribed in many studies. In the present work we tested the ability of T-cell clones to release TNFs and IFNs before and after mitogen stimulation. Some clones from the patients and none from the controls released the inhibitors (IFNs) without requiring in vitro stimulation. After mitogen priming a higher percentage of patients' T-cell clones was able to release TNFα and/or IFNs in greater mean quantities as compared with controls (Table 3). This result confirms the possibility of an overproduction of such lymphokines in SAA patients. IFN- and TNFα-producing T-cell clones were above all CD8+. Thus, CD8+ cells, which are expanded both in PB and BM of SAA patients, seem to be also suppressor clones, especially because TNFα and IFNs may act synergistically in the inhibition of growth and differentiation of granulocyte and monocyte precursor cells. Furthermore, the same CD8+ clones producing high quantities of IFNs and TNFα seemed to produce less GM-CSF than controls, at least after priming with PMA + TPA.

We also found in patients greater percentages of PB and BM T-cell microcultures able to release IL-2 after mitogen stimulation (data not shown). Although the differences observed were not statistically significant, possibly due to the relatively low number of clones tested, this finding seems to confirm the observations of Gascon et al on the increased ability of T cells of SAA patients to release IL-2 after mitogen stimulation. As known IL-2 is a lymphokine that plays a crucial role in the development of immune responses and the great number of T cells able to produce it, may suggest again a state of immune activation.

In conclusion, T-cell clones from SAA patients exhibit predominantly a CD8+ phenotype, a greater cytotoxic activity, and can be shown to produce greater quantities of suppressor lymphokines when compared with normal controls.

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REFERENCES


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