Analysis of Natural Killer Cells in Patients With Aplastic Anemia

By Pedro Gascón, Nicholas Zoumbos, and Neal Young

We have analyzed natural killer (NK) cells in 43 patients with severe aplastic anemia, using cytotoxicity assays and microfluorometry with monoclonal antibodies, prior to and after treatment with antithymocyte globulin (ATG). Before treatment, natural killer cell activity (NKa) in both peripheral blood and bone marrow was markedly decreased in 76% of patients as compared with normal controls. Although we have measured low NKa in patients receiving large numbers of blood transfusions (x̄ = 150 U of RBCs), six aplastic patients had low NKa in the absence of transfusions, and the average number of transfusions in the total population was low (x̄ = 24). Purification of larger granular lymphocytes (LGLs) from peripheral blood of aplastic anemia patients failed to recover significant NKa. Most of these large granular lymphocytes showed few azurophilic granules. NKa was appropriately enhanced in these patients’ samples by exposure of mononuclear cells to either interleukin 2 (IL-2) or interferon (IFN). Analysis of peripheral blood phenotypic markers showed that cells bearing Leu 7 antigen were in the normal range in aplastic anemia (x̄ = 12% ± 2%; normal = 16% ± 2%), but there was a deficiency of Leu 11+ cells (x̄ = 8% ± 2%; normal = 15% ± 2%). The number of Leu 11+ cells was well correlated with NKa. In 13 of 22 patients treated with ATG, NKa returned to the normal range, and recovery of NKa was correlated with hematopoietic recovery. Our results suggest that deficient NKa is an intrinsic feature of aplastic anemia, and that the circulating cells in this disease are of the pre-NK cell stage.

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Materials and Methods

Study Population

Forty-three patients (mean age 34 ± 16 years) with severe aplastic anemia, as defined by peripheral blood counts and bone marrow biopsy,28 were studied. Twenty-three patients had acute disease (less than three months from the time of diagnosis to study), and 20 had chronic disease. All patients were studied at least once for natural cytotoxic activity, and assays in ten patients were repeated twice prior to antithymocyte globulin (ATG) treatment. Tests were carried out at admission and at 30, 60, 90, and 180 days after completion of treatment. Thirty-seven patients had received some platelet or erythrocyte transfusions prior to the study. None of the patients showed clinical evidence of active viral infection, and their titers of antibodies to Epstein-Barr virus, cytomegalovirus, and hepatitis A and B virus were lower than were those of multiply transfused patients.27 Sixty-four normal laboratory personnel (mean age 31 ± 8 years) of three races served as controls.

Preparation of Cells

Peripheral blood lymphocytes. Fresh blood was drawn into syringes, containing 10 U/mL of preservative-free heparin (O’Neill and Feldman, St Louis), and mononuclear cells were separated on Ficoll-Hypaque (LSM, Litton Bionetics, Kensington, Md) gradients. Cells were washed three times and resuspended in RPMI 1640 medium (M. A. Bioproducts, Walkersville, Md) supplemented with 2 mmol/L of glucose, 100 U/mL penicillin/streptomycin (M. A. Bioproducts), and 10% heat-inactivated fetal calf serum (FCS) (Hyclone, lot 100378, Sterile Systems, Inc, Logan, Utah).

Lymphocytes were purified by two-step adherence-nonadherence separation, on plastic surfaces and nylon wool columns, and sedimentation on Percoll gradients. Low-density cells, which are mostly LGLs, and high-density cells (predominantly T lymphocytes) were obtained by centrifugation on a discontinuous gradient of Percoll (Pharmacia, Uppsala, Sweden).28 An essential step before the gradient centrifugation was the rigorous removal of adherent cells that tended to band in the fractions close to those of LGLs. Almost complete removal of adherent cells was achieved by incubation on plastic Petri dishes of mononuclear cells obtained after Ficoll-Hypaque centrifugation (3003; Falcon Labware, Division of Becton Dickinson, Oxnard, Calif) for one hour at 37°C and a further

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incubation on nylon columns (Associated Biomedic Systems, Inc, Buffalo) for 30 minutes at 37 °C. Nonadherent cells were applied to a seven-layer discontinuous Percoll density gradient that varied in concentration from 2.5% to 40%–55%. Cells from all layers were examined by morphology using Giemsa-stained cytopreps. LGLs were recovered from fractions II and III at 42.5% to 45% Percoll.

Bone marrow lymphocytes. Bone marrow was aspirated into 1 mL of Iscove's modification of Dulbecco's medium (IMDM) and 250 U of preservative-free heparin. Bone marrow mononuclear cells were obtained by the same procedure as is used to obtain peripheral blood. Informed consent was obtained from patients and normal subjects according to protocols approved by the NHLBI review board.

Flow Cytometry

T lymphocyte subpopulations from peripheral blood mononuclear cells and from Percoll fractions I, II, III, and IV were quantitated by direct immunofluorescence using either fluorescein-conjugated or phycoerythrin-conjugated monoclonal antibodies Leu 1, Leu 2, Leu 3, Leu 7, Leu 11a, and anti-HLA-DR (nonpolymorphic, clone L243) (Becton Dickinson, Sunnyvale, Calif); mouse-IgG conjugated to the appropriate fluorescent molecule served as a negative control. T lymphocytes were suspended in Hank's balanced salt solution (HBSS) without phenol red (M. A. Bioproducts), containing 0.1% sodium azide and 0.1% bovine serum albumin (BSA). After centrifugation, 1 x 10^6 cells were incubated with 5 μL of appropriate monoclonal reagent at 4 °C for 30 minutes and then washed twice. Double labeling was used, a second monoclonal antibody conjugated either with fluorescein or with phycoerythrin was added for 30 minutes at 4 °C; the cells were then washed twice. Flow microfluorometry was performed using a FACS II (Becton Dickinson FACS System); data were selected, stored, and analyzed using a PD 11/35 computer (Digital Equipment Corp, Marlboro, Mass).

Cell sorting was performed in some experiments using the NK cell markers Leu 7 and Leu 11a. Morphology of 200 sorted cells was then examined, using Giemsa-stained cytopreps, by one of the authors and an independent hematologist.

NK Cell Assay

Natural killer cell activity (NKa) was assayed by release of 51chromium (51Cr) from K562 target cells. Target cells were labeled with 200 μCi of 51Cr (Amersham, Arlington Heights, Ill) for one hour at 37 °C and were washed extensively before use. Target cells, 5 x 10^3 51Cr-labeled K562 in 100 μL of medium, were dispensed into 96-well, round-bottom microtiter plates (Costar, Cambridge, Mass). Mononuclear effector cells (100 μL) were added to wells in triplicate at serial effector-target cell ratios ranging from 100:1 to 6.25:1. One hundred microliters of supernatant was harvested for five hours at 37 °C in a 5% CO2 humidifier chamber, labeled target cells were added to the respective wells. Supernatants were harvested four hours later and assayed as described above.

Statistical analysis. Data were analyzed using Student's t test, the two-tailed nonparametric Wilcoxon test, the Fisher exact two-tailed test, and linear regression analysis.

RESULTS

NKa in peripheral blood. NKa in the blood of most patients with aplastic anemia was markedly reduced in comparison to that of normal individuals (P < .002). Titration at different effector-target ratios in the first 38 patients studied are shown in Fig 1; of these 38 patients, 18 had no detectable NKa; 11 other patients had activity <2 SD from the normal mean. Cytotoxicity for K562 leukemic cell targets increased with time, i.e., from 4 to 18 hours of incubation, but did not reach the level of normal controls at any given time at effector-target ratios ranging from 100:1 to 6.25:1. NKa in acute aplastic anemia patients (n = 18; x ± SE = 11 ± 3) was not statistically different from chronic patients (n = 20; x ± SE = 19 ± 4). To determine if decreased NKa was due to the presence of a soluble factor in the circulation of patients with aplastic anemia, sera from six patients was preincubated for one hour at 37 °C with normal peripheral blood mononuclear cells (PBMNCs) before addition of target cells; under these circumstances, NKa of the normal cells was unaffected (data not shown).

NKa in bone marrow. Because NK cells have been suggested as possibly mediating hematopoietic suppression in aplastic anemia, we measured NKa in the bone marrow of 12 patients. Measurement of a low proportion of total and helper T cells in the bone marrow as compared with the peripheral blood suggested that dilution of the marrow samples by peripheral blood was minimal. As shown in Fig 2, mean NKa in aplastic bone marrow was decreased as compared with normal and to approximately the same degree.

![Fig 1. Peripheral blood natural killer cell activity (NKa) in aplastic anemia patients, n = 38 (o) as compared with normal controls, n = 60 (o): (A) at four hours (P < .0001) of incubation; (B) at 18 hours of incubation (P < .006). Bars represent SE.](www.bloodjournal.org)
NK CELLS IN APLASTIC ANEMIA

Although the same number of LGLs were tested for activity as in normal controls, the LGLs in aplastic anemia had defective NK<sub>a</sub>. In only one case, in which NK<sub>a</sub> in unfractionated cells was in the low normal range, was normal activity recovered following Percoll gradient centrifugation.

**LGL morphology.** The light-microscopy morphology of LGLs from patients with aplastic anemia appeared abnormal (Fig 3) in that they showed a marked lack of granules in most of the LGLs in comparison with normal LGLs. In preparations from normal individuals, >90% of LGLs showed prominent azurophilic granules, and the remaining cells showed fine granulation. In contrast, in preparations from aplastic anemia patients, >20% of LGLs lacked granules, >40% had fine granulation, and the remaining cells showed only a few granules. These data suggest that NK cells were qualitatively as well as numerically defective in aplastic anemia.

**Analysis of phenotypic markers.** Two monoclonal antibodies, termed Leu 7 and Leu 11, have been described to bind with some degree of specificity to NK cells, with Leu 11-bearing cells being the more effective NK cells in functional assays. To assess the NK cell phenotypes that were present in aplastic anemia, flow microfluorometry of surface antigens was performed on PBMCs of 29 patients with aplastic anemia and 19 normal controls. The mean proportion of cells bearing the Leu 7 antigen in the patient population was not significantly different from normal (Table 2), although 30% of patients had much higher levels of Leu 7<sup>+</sup> cells than did normal subjects. There was no positive correlation between the number of Leu 7<sup>+</sup> cells and NK<sub>a</sub> in aplastic anemia patients (r = .1) (Fig 4) such as was found for the normal controls (r = .4). In contrast, the number of Leu 11-bearing cells in aplastic anemia correlated to NK<sub>a</sub> (r = .64) as it did among normals (r = .52) (Fig 4). The absolute numbers of both Leu 7<sup>+</sup> and Leu 11<sup>+</sup> cells in the circulation of patients with aplastic anemia were markedly decreased (P < .05).

In three patients, cells from Percoll gradient layers I, II, III, and IV were analyzed for NK cell markers (Table 3). There was low NK<sub>a</sub> in all fractions, which correlated with the low proportion of Leu 11<sup>+</sup> cells, analogous to the results in peripheral blood.

**Table 1. Effect of Lymphokine Stimulation on NK<sub>a</sub> in Aplastic Anemia**

<table>
<thead>
<tr>
<th>Source of Effector Cells</th>
<th>Percentage of NK&lt;sub&gt;a&lt;/sub&gt;</th>
<th>4-h Incubation</th>
<th>18-h Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium 100:1</td>
<td>+ IL-2 100:1</td>
<td>+ IFN 100:1</td>
</tr>
<tr>
<td>Peripheral blood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NC</td>
<td>51 ± 1 (60)</td>
<td>61 ± 4 (15)</td>
<td>67 ± 4 (15)</td>
</tr>
<tr>
<td>AA</td>
<td>15 ± 2 (38)</td>
<td>42 ± 5 (15)</td>
<td>39 ± 6 (16)</td>
</tr>
<tr>
<td>P†</td>
<td>&lt;.0001</td>
<td>&lt;.02</td>
<td>&lt;.002</td>
</tr>
<tr>
<td>Bone marrow</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NC</td>
<td>32 ± 4 (12)</td>
<td>49 ± 5 (8)</td>
<td>54 ± 5 (8)</td>
</tr>
<tr>
<td>AA</td>
<td>14 ± 2 (16)</td>
<td>24 ± 5 (12)</td>
<td>23 ± 5 (12)</td>
</tr>
<tr>
<td>P†</td>
<td>&lt;.002</td>
<td>&lt;.01</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

NK<sub>a</sub>, natural killer cell activity; NC, normal controls; AA, aplastic anemia patients.

Results are given as mean ± SE. Number of patients studied in parentheses.

*Effect-target ratio.

†Two-tailed P value using the nonparametric Wilcoxon test.
Table 2. Phenotype Markers for NK in PBMNCs of Aplastic Anemia Patients

<table>
<thead>
<tr>
<th>Marker</th>
<th>Pre-ATG</th>
<th>Post-ATG</th>
<th>Normal Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu-7 (%)</td>
<td>12 ± 2 (29)</td>
<td>21 ± 7 (10)*</td>
<td>16 ± 2 (19)</td>
</tr>
<tr>
<td>Leu-11 (%)</td>
<td>8 ± 2 (20)</td>
<td>12 ± 6 (10)</td>
<td>15 ± 2 (10)</td>
</tr>
<tr>
<td>DR-Leu-3/DR-Leu-2 (%)</td>
<td>0.9 ± 0.2 (15)</td>
<td>0.8 ± 0.1 (10)</td>
<td>4.2 ± 4 (10)</td>
</tr>
</tbody>
</table>

NK, natural killer cells; PBMNCs, peripheral blood mononuclear cells; ATG, antithymocyte globulin.

Results are given as mean ± SE. Number of patients studied in parentheses; %, percentage of total peripheral blood lymphocytes positive.

*Three responders and seven nonresponders, one studied 1 month and nine studied 3 months after ATG therapy.

activated helper-suppressor lymphocyte ratio \( r = .79 \), which was defined by two-color fluorometry using anti-HLA-DR monoclonal antibody as a marker of T cell activation (DR-Leu 3/DR-Leu 2) (Fig 4). A similar relationship existed for normal controls \( n = 10 \), in whom the helper-suppressor lymphocyte ratio highly correlated with NK\(_a\) \( r = .86 \) as did the activated helper-suppressor lymphocyte ratio \( r = .98 \).

Effect of therapy on NK\(_a\). Between 40% and 50% of patients with aplastic anemia showed a hematopoietic response to therapy with ATG. 3 To determine the effects of ATG on recovery of NK\(_a\), we prospectively studied NK\(_a\) in 22 patients prior to and following ATG therapy (Fig 5). In this series, 13 patients became transfusion-independent. NK\(_a\) increased into the normal range in a total of 13 patients; in four patients NK\(_a\) decreased following therapy, and in five patients it remained unchanged. There was a strong correlation between hematopoietic recovery and return to normal of NK\(_a\); 11 of 13 patients who responded hematopoietically recovered normal NK\(_a\) \( P < .01 \) by the Fisher exact test) (Table 4). However, in one patient who had a return to normal blood counts, NK\(_a\) remained absent. NK\(_a\) increased in two of nine patients who failed to respond hematologically to ATG treatment.

DISCUSSION

NK\(_a\) in the blood of patients with aplastic anemia was markedly reduced in the majority of the 38 patients we now report and in a previously published series. 32 We also provide

Fig 3. Wright's Giemsa cytospin preparation of stained cells from fractions II and III of Percoll gradients. (A) Large granular lymphocytes (LGLs) from a normal control; (B) LGLs from an aplastic anemia patient. Both cell preparations were assayed simultaneously. Aplastic anemia LGLs showed few azurophilic cytoplasmic granulations. One experiment representative of eight is shown.

In normal individuals, cells bearing Leu 11 outnumber those bearing Leu 7 \( (\tilde{x} \text{ ratio} = 1.35 ± 0.28) \). However, in the aplastic population, Leu 7 cells predominated \( (\tilde{x} \text{ ratio} = 0.85 ± 0.53) \) and ratios of 11 of 21 patients were below 2 SD from the normal mean. In addition, we also assessed the phenotypes of T cells in PBMNCs of aplastic anemia patients. NK\(_a\) was directly correlated \( r = .62 \) to the relative proportion of T helper and T suppressor cells (Leu 3/Leu 2) (data not shown). NK\(_a\) had a somewhat better correlation to the

Fig 4. Natural killer (NK) cell phenotypic markers in peripheral blood mononuclear cells from aplastic anemia patients: (A) Leu-7 plotted against NK\(_a\) \( r = .11 \); (B) Leu 11 correlated with NK\(_a\) \( r = .64 \); (C) correlation between the activated helper-suppressor lymphocyte ratio and NK\(_a\) \( r = .79 \). NK\(_a\) is expressed as percentage of cytotoxicity at an effector-target ratio of 100:1.
new evidence that correspondingly low NK\(a\) also exists in bone marrow mononuclear cells obtained from patients with aplastic anemia. These results suggest that defective NK function is a consequence of the underlying bone marrow failure and therefore do not support the suggestion\(^{31,32}\) that hematopoietic suppression in aplastic anemia is mediated by NK cells, at least as these cells are functionally defined. Abnormal IFN production\(^{25}\) and activated suppressor bym-

**Table 3. NK\(a\) and Surface Phenotype of Percoll Gradient Fractions**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Fraction No.</th>
<th>Fraction I</th>
<th>Fraction II + III</th>
<th>Fraction IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK(a) (%)</td>
<td>AA</td>
<td>0</td>
<td>12 ± 9</td>
<td>5 ± 5</td>
</tr>
<tr>
<td></td>
<td>NC</td>
<td>10 ± 2</td>
<td>78 ± 10</td>
<td>30 ± 4</td>
</tr>
<tr>
<td>Leu-7 (%)</td>
<td>AA</td>
<td>20 ± 4</td>
<td>48 ± 6</td>
<td>26 ± 11</td>
</tr>
<tr>
<td></td>
<td>NC</td>
<td>14 ± 2</td>
<td>44 ± 10</td>
<td>19 ± 8</td>
</tr>
<tr>
<td>Leu-11 (%)</td>
<td>AA</td>
<td>4 ± 2</td>
<td>18 ± 8</td>
<td>8 ± 3</td>
</tr>
<tr>
<td></td>
<td>NC</td>
<td>18 ± 4</td>
<td>67 ± 6</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>Leu 1 (%)</td>
<td>AA</td>
<td>ND</td>
<td>22 ± 3</td>
<td>92 ± 7</td>
</tr>
<tr>
<td></td>
<td>NC</td>
<td>ND</td>
<td>14 ± 6</td>
<td>88 ± 6</td>
</tr>
</tbody>
</table>

AA, aplastic anemia (n = 3); baseline natural killer cell activity for AA (NK\(a\)) = 8% ± 12%. NC, normal controls (n = 3); baseline NK\(a\) for NC = 42% ± 11%. ND, not done (insufficient cells for analysis by flow cytometry). Results are expressed as (X ± SEM).

**Table 4. Effect of Post-ATG–NK\(a\) v Hematopoietic Recovery**

<table>
<thead>
<tr>
<th>NK(a)</th>
<th>Increased*</th>
<th>No Change or Decreased</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR</td>
<td>+</td>
<td>11</td>
</tr>
<tr>
<td>-</td>
<td>2</td>
<td>7</td>
</tr>
</tbody>
</table>

Natural killer cell activity (NK\(a\)) was determined one or three months after beginning of treatment and compared with values obtained before therapy. The results were highly significant by the Fisher two-tail test (P < .01). HR, hematopoietic response.

*Defined as post-ATG value > 30% higher than pre-ATG value.

tive defect, either a deficiency in the number of NK cells produced or the ability of NK cells to function as measured in the K562 cell cytotoxicity assay. The pancytopenia that characterizes aplastic anemia probably encompasses more than erythrocytes, platelets, and granulocytes. For example, monocytes,\(^{38}\) B lymphocytes,\(^{36}\) and helper/inducer lymphocytes\(^{33}\) also have been reported to be decreased in these patients. NK cells have been assigned to both monocytic\(^{37}\) and lymphocytic\(^{38}\) lineages. We were unable to recover normal numbers of LGLs by density gradient fractionation of mononuclear cells, suggesting that NK cells in fact are decreased in number in aplastic anemia. The very low absolute numbers of Leu 7 and Leu 11 antigen-bearing mononuclear cells in patients with aplastic anemia indicates that NK cell numbers are reduced as a function of the pancytopenia that characterizes aplastic anemia. In addition to the reduced number of NK phenotypes, the NK cells with LGL morphology showed defective cytotoxic function. The recovery of hematopoietic function in treated aplastic anemia patients generally was associated with an increase in NK\(a\) and NK cell phenotype.

In spite of impairment in spontaneous NK\(a\), aplastic anemia patients have lymphokine-responsive NK cells in both peripheral blood and bone marrow. These cells readily responded to activation by IFN or IL-2 in vitro, and NK\(a\) could be reconstituted to the normal spontaneous range by either lymphokine. Therefore, lymphokine-responsive NK cells are intact in aplastic anemia, although there is a deficiency of cells with spontaneous NK\(a\).

Phenotypic analysis of residual NK cells in the peripheral blood of aplastic anemia patients showed that there was a relative predominance of Leu 7* cells and a marked reduction in the number of Leu 11* cells. Leu 11*, Leu 7* NK cells have been characterized as having the highest spontaneous NK\(a\).\(^{39,40}\) Our data suggest that most cells bearing the NK phenotype in aplastic anemia may be characterized instead as lymphokine-responsive NK precursor cells with low spontaneous NK\(a\). The small number of granules in LGLs isolated from aplastic anemia patients also indicates that most of the cells are in an inactive form, possibly representing the precytotoxic stage.\(^{39,40}\) The results are all consistent with an immature but lymphokine-responsive population of NK cells contributing to the low NK\(a\) measured in blood and bone marrow.

How can low NK\(a\) be related to the abnormalities of lymphokine production in aplastic anemia? INF and IL-2 stimulate NK\(a\), and the production of these two lymphokines

**Fig 5. The effect of antithymocyte globulin (ATG) therapy on natural killer cell activity (NK\(a\)) with (n = 13) (A) and without (n = 9) (B) hematologic recovery. NK\(a\) is expressed as percentage of cytotoxicity at an effector-target ratio of 100:1. Blood counts at three months after ATG therapy showed: Absolute reticulocytes (x10\(^{12}\)/\(\mu\)L) for A were 5.5 ± 3; for B, they were 2.6 ± 1.2 (with transfusion support). Absolute neutrophils (—/\(\mu\)L) for A were 1211 ± 901, and for B were 742 ± 700. Platelets (x10\(^{12}\)/\(\mu\)L) for A were 43 ± 52, and for B were 9 ± 4.**
by PBMCs in vitro is very high; serum levels of IFN are also elevated in 30% of patients with aplastic anemia and in the bone marrow of almost all cases. One explanation for this possible discordance is the differing effect of short-term and long-term exposure to IFN in vivo. Infusion of IFN in cancer patients increases NK$_L$ during the first 24 hours of administration, but thereafter NK$_L$ is low. In addition, acquired immunodeficiency syndrome (AIDS) patients who reportedly have elevated circulating IFN levels are defective in NK function, similar to aplastic anemia patients. The consistent association of high IFN levels and low NK$_L$ in vivo suggests the existence of a feedback regulation mechanism that controls the expression of activity in NK cells.

The functional significance of low NK$_L$ in aplastic patients is uncertain. Long-term monitoring of NK$_L$ in patients undergoing ATG therapy, however, indicates that recovery of NK function correlates well with response to therapy.

Patients with aplastic anemia are not abnormally susceptible to viral infections. Although leukemia develops in a small percentage of patients with primary bone marrow failure, the prognosis of this group of patients with severe disease is too poor to allow study of malignant transformation over long periods of time. Nevertheless, the existence of pre-NK cells responsive to lymphokine activation suggests that potentially there exists a reserve of NK cells that might, under appropriate stimulation, confer antiviral or antitumor resistance.

Observation of patients who recover hematopoietic function with persistent low NK$_L$ and of patients with other diseases associated with low NK$_L$ may permit better definition of a physiologic role in humans for these cells.

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