Impaired Natural Killer Activity in Patients With Chronic Lymphocytic Leukemia Is Associated With a Deficiency of Azurophilic Cytoplasmic Granules in Putative NK Cells

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This study was undertaken to gain further insight into the severely impaired natural killer (NK) activity we and others have previously observed in patients with chronic lymphocytic leukemia (CLL). Normal individuals' NK cells are large granular lymphocytes (LGL) that (A) bind to and lyse NK-sensitive cells, including K562, (B) express receptors for the Fc portion of IgG (FcR⁺ cells), and (C) express cell surface antigens reactive with monoclonal antibodies OKM1, 9.6, and OKT11A. We thus examined lymphocytes depleted of monocytes and B cells, from 6 CLL patients and 6 normal individuals, that were identified on the basis of binding to K562, expressing OKM1, or expressing receptors for the Fc portion of IgG. In the CLL patients studied, lymphocytes that bind to K562 cells, as well as OKM1⁺ cells isolated by fluorescence activated cell sorting, were morphologically similar to LGL of normal individuals, with the exception that more than 75% of the patients' cells were deficient in azurophilic cytoplasmic granules, which typify normal individuals' LGL. Furthermore, although the percentages of the patients' FcR⁺ cells reactive with OKT11A, 9.6, and OKM1 were very similar to those of normals, the majority of the patients' FcR⁺ cells were deficient in azurophilic granules and lacked NK activity. These findings indicate that the impaired NK activity in CLL patients is associated with cells that are phenotypically and morphologically NK cells, but which lack azurophilic granules that are thought to play a role in NK-mediated lysis.

WE AND OTHERS have reported that patients with the common form of B cell chronic lymphocytic leukemia (CLL) have severely diminished natural killer (NK) cell activity compared to that of normal individuals. This finding has been made using peripheral blood lymphocytes (PBL) depleted of B cells by either isolating lymphocytes that rosette with sheep red blood cells (E) or by treatment with a monoclonal anti-B cell antibody and complement. Despite the extremely low level of NK cell activity in CLL patients, we observed that the proportion of the B cell-depleted PBL of the patients that bind to NK-sensitive targets, such as K562 cells, falls within the range of that of normal individuals. In addition, a larger than normal proportion of E rosetting cells from CLL patients express receptors for the Fc portion of IgG (referred to here as FcR⁺ cells), a characteristic of normal NK cells.

More recently, we and others have further characterized human NK cells with regard to expression of cell surface antigens reactive with monoclonal antibodies. Most NK cells express antigens reactive with monoclonal antibodies OKM1 and OKT11A (or its homologue, 9.6), and in addition, nearly all NK activity resides within a population of cells referred to as large granular lymphocytes (LGL), which generally have indented nuclei and azurophilic cytoplasmic granules. We have undertaken studies aimed at determining whether the deficient NK activity in CLL patients may be associated with a lack of cells with the properties of normal NK cells. We report that the vast majority of monocyte- and B cell-depleted lymphocytes from CLL patients that either (A) bind to K562 target cells, (B) are FcR⁺, or (C) express OKM1 are morphologically similar to LGL of normal individuals but, in contrast to the normals' cells, most of the patients' cells are deficient in the azurophilic cytoplasmic granules that are thought to play a role in NK-mediated lysis.

MATERIALS AND METHODS

Patients

Patients with chronic lymphocytic leukemia (CLL) who have been followed at the Veterans Administration Medical Center, Minneapolis, MN, and who were not on therapy for at least 3–4 wk were studied following provision of informed, written consent. The patients chosen had been found to have very low or no detectable NK activity mediated by their B cell-depleted PBL.

Depletion of Monocytes and B Cells From Peripheral Blood

Peripheral blood mononuclear cells were isolated from heparinized blood by Ficoll-Hypaque sedimentation, and the monocytes were depleted by two cycles of adherence to plastic flasks in RPMI 1640, containing 10% heat-activated fetal calf serum (FCS). This medium will be referred to as "culture medium." The nonadherent peripheral blood lymphocytes (PBL) were harvested and depleted of B cells by either of the following two methods: (1) incubation of the PBL with 2-amino ethylisothiouronium bromide hydrobromide

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(AET) treated sheep red blood cells (E) and then isolation of the E-rosetting cells on Ficoll-Hypaque gradients, or (2) treatment of the PBL (5 × 10^7) with monoclonal antibody BA-1 (kindly provided by Dr. T. LeBien, University of Minnesota, Minneapolis, MN), which reacts with normal and malignant B cells and complement (C), as previously detailed. These cells were then washed twice and resuspended in RPMI 1640 containing 10% fetal calf serum (FCS). Following treatment with BA-1 and C, less than 1% of the cells expressed surface immunoglobulin as detected by immunofluorescence.

**Cell Binding Studies**

Lymphocytes were depleted of monocytes and B cells as described above and were allowed to form conjugates with K562, as previously detailed.1

**Isolation of Cells Expressing Receptors for the Fc Portion of IgG (FcR Cells)**

E rosetting cells were isolated as detailed above, and FcR+ cells were separated from the FcR- cells using ox RBC coated with anti-ox IgG, as previously described.15

**Cell Surface Marker Analysis**

Monoclonal antibody OKM1, which reacts with monocytes and 7%-15% of nonadherent peripheral mononuclear cells,13 including most large granular lymphocytes (LGL),14 was kindly provided by Dr. Patrick Kung. Monoclonal antibody OKT11A (also provided by Dr. Patrick Kung) and monoclonal antibody 9.6 (kindly provided by Dr. John Hansen) react with all E-rosetting cells.15,16 Lymphocytes were incubated with the optimal dilution of the antibodies for 30 min on ice. The cells were washed twice and incubated with a 1:10 dilution of fluorescein isothiocyanate (FITC) conjugated F(ab')2 goat anti-mouse IgG (Cappel Labs, Cochranville, PA) for 30 min on ice. After thorough washing, cells were examined under a Zeiss fluorescent microscope.

**Isolation of OKM1+ and OKM1− PBL by Fluorescence Activated Cell Sorting**

OKM1+ and OKM1− cells were separated from peripheral blood mononuclear cells that had been depleted of monocytes by plastic adherence and depleted of B cells by treatment with BA-1 plus C, as detailed above. The dead cells were removed by Ficoll-Hypaque gradient centrifugation, and the cells were washed thoroughly and cultured overnight in RPMI 1640 supplemented with 10% human serum. The cells were washed twice and then incubated with 0.5 ml of a 1:50 dilution of OKM1 for 30 min on ice. After being washed twice, the cells were washed again with a 0.15 ml of a 1:10 dilution of FITC-conjugated F(ab')2 goat anti-mouse IgG for 30 min on ice. After thorough washing, the cells were resuspended in PBS containing 3% human serum and were sorted on a FACS IV (Beckton-Dickinson, Mountain View, CA). Cells that were reacted with FITC-goat anti-mouse IgG alone served as the negative control for calibrating the FACS IV.

**31Cr Release Assays**

The NK-sensitive cell line K562 was labeled for 1 hr with 100 μCi Na₂³¹CrO₄, New England Nuclear, Boston, MA), washed 3 times, and adjusted to 10⁷ cells/ml in culture medium. Then, 0.1 ml of threefold serial dilutions of effector cells and 0.1 ml target cells were admixed in triplicate round-bottom wells of Linbro plates (Linbro, Hamden, CT). Triplicate wells with target cells in medium alone or in detergent were included to determine spontaneous and total release, respectively. Plates were spun for 5 min at 100 rpm and incubated for 16 hr at 37°C in a humidified CO₂ atmosphere. Then, 0.1 ml of supernatant was removed from each well and counted in a gamma counter. The percent specific ³¹Cr released from the target cells was calculated by the formula:

\[
\text{Experimental release} - \text{Spontaneous release} \times 100
\]

**Assay for ADCC**

The antibody-dependent complement-mediated cytotoxicity (ADCC) activity of normal and CLL effector cells (FcR+) was determined in the following manner. FcR+ isolated cells (as described above) were allowed to incubate for 1-2 hr after isolation procedure. This is done because freshly isolated normal FcR+ cells have significantly reduced ADCC activity. We have noted, however, that a 1-2-hr intravenous incubation of the FcR+ cells results in easily detectable ADCC function. Then, 50 μl of varying concentrations of effector cells were added to 50 μl of culture medium containing ³¹Cr-labeled Raji target cells (la positive) and 100 μl of a 10⁻⁴ dilution of mouse monoclonal anti-la antisera, which was previously found to yield maximal ADCC. The mixtures were incubated for 4-6 hr at 37°C in 5% CO₂. The ADCC activity was calculated as described above for NK activity.

**RESULTS AND DISCUSSION**

Results suggest that the impaired NK cell activity we and others have previously detected in patients with CLL,1,2 is related to the presence of putative NK cells that are defective by virtue of a deficiency in the cytoplasmic azurophilic granules that typify normal individuals' NK cells. The latter statement is based on three separate findings comparing monocyte and B cell-depleted cells from CLL patients and normal individuals. First, most of the K562-binding cells of patients and normals are morphologically similar, except that the majority of the patients' K562-binding cells had few, if any, cytoplasmic granules. Results shown in Fig. 1A reveal that the E-rosetting cells from CLL patients that bind to K562 are approximately the same size as those of normal individuals (Fig. 1B) and are similar to large granular lymphocytes (LGL), which characterize normal NK cells.8 However, there is a definite decrease in the number of obvious azurophilic granules in the cytoplasm of the patients' lymphocytes as compared to the normals' K562-binding cells. The figures shown are typical of the observations made on four different CLL patients and normal individuals.

Second, since only a portion of PBL that bind to K562 have cytotoxic activity,17 we questioned whether CLL patients' cells that express the OKM1 cell surface marker, which is present on at least 75% of normal individuals' cells with NK activity and LGL morphology, would likewise resemble LGL but be deficient in the azurophilic granules. Figures 1C and 1D are representative of the morphology of the OKM1+ cells isolated by fluorescent-activated cell sorting from
Fig. 1. Morphology of K562-binding cells and OKM1\(^+\) and OKM1\(^-\) cells. Wright-Giemsa-stained cytocentrifuge preparations (x 340). (A) Normal donor's E-rosetting cells binding to a K562 cell. Note that most of the K562-binding cells contain multiple cytoplasmic granules. (B) CLL patient's E-rosetting cells binding to a K562 cell. Note the absence of obvious cytoplasmic granules in most of the binding cells. (C) OKM1\(^+\) cells isolated from monocyte- and B cell-depleted cells of a normal donor. Note the presence of multiple cytoplasmic granules. (D) OKM1\(^+\) cells isolated from a CLL patient. Note that the majority of the cells have few, if any, cytoplasmic granules. (E) OKM1\(^-\) cells isolated from a normal donor. The OKM1\(^+\) cells from the CLL patients were morphologically the same.
monocyte-depleted PBL of normal individuals and CLL patients. OKM1+ cells from the CLL patient (Fig. 1D) are markedly deficient in cytoplasmic granules as compared to the OKM1+ cells from the normal individual (Fig. 1C). In the four normal individuals studied, 86%-90% of the OKM1+ cells isolated by the FACS IV had two or more azurophilic granules, with 8%-10% of the cells containing 0–1 granule. Our findings confirm previous reports that most OKM1+ cells are LGL.9 In contrast, in the four CLL patients studied, only 10%-20% had 2 or more granules, 2%-4% had 1 single visible granule, and 75%-81% of the OKM1+ cells had no obvious granules. The OKM1+ cells from both the patients and normals were otherwise similar with regard to the size of the cells, nuclear/cytoplasmic ratio, and nuclear morphology. The OKM1- cells were homogeneous, in that the vast majority were typical small lymphocytes with a high nuclear-to-cytoplasmic ratio and an agranular cytoplasm. Figure 1E depicts normal individuals’ OKM1- cells. The OKM1- cells from CLL patients appeared morphologically identical.

As it has been shown that, in normal individuals, most NK cells express receptors for the Fc portion of IgG (FcR+ cells), we compared the morphology, cytotoxic activity, and phenotype of the FcR+ cells isolated from the monocyte and B cell-depleted mononuclear cells of normal individuals and CLL patients. The morphology of the FcR+ cells from normals was essentially the same as that of the OKM1+ cells in that they were primarily LGL, whereas the FcR+ cells of the patients resembled the patients’ OKM1+ cells (shown in Fig. 1D), in that most had one or less than two visible cytoplasmic granules. In normals, most NK activity resides with the FcR+ population.1 We thus compared the cytotoxic activity mediated by the FcR+ cells from normals and patients. Results, presented in Table 1B, show that, whereas FcR+ cells from two normal individuals were cytotoxic against 51Cr-labeled K562 targets, the FcR+ cells isolated from the two CLL patients had minimal, if any, NK activity. The FcR+ cells from the patients lacked detectable NK activity, and those of normals had markedly reduced NK activity compared with their FcR+ cells (data not shown). The phenotype of the FcR+ cells isolated was tested in order to determine whether they express the same membrane antigens as those expressed on normal individuals’ FcR+ cells. Results (Table 1A) show that more than 85% of the FcR+ cells from the normals and patients were OKM1+, as previously reported in normal individuals.22 The vast majority of the patients’ and normals’ E rosetting FcR+ cells also reacted with monoclonal antibodies, 9.6 and OKT11A, which appear to be directed against the E receptor.15,16 It is thus apparent that the FcR+ cells from the patients are phenotypically the same as those of normals in regard to reactivity with monoclonal antibodies that characterize normal NK cells, but the FcR+ cells of the patients lack cytotoxic activity and are deficient in cytoplasmic azurophilic granules. These data are consistent with, but do not prove, that the CLL cells we are examining are NK cells.

The relative absence of cytoplasmic granules in the putative NK cells of CLL patients suggests that the inability of CLL patients’ cells to exert NK activity may be related to this finding. Evidence that the cytoplasmic granules play a role in cytolysis derives from the following findings. LGL lose their cytotoxic activity following strontium-induced degranulation, and a recovery of NK function following this degranulation was found to correlate with the reacquisition of the intracytoplasmic azurophilic granules.9,10 Second, electron microscopic studies have shown that granules may be released from LGL during lysis of K562 cells (Dr. Barry Bloom, personal communication). Third, it has recently been reported that there are two populations of peripheral blood lymphocytes expressing the antigen detected with monoclonal antibody HNK-1 that reacts with most NK cells; one HNK-1+ population exhibited a high level of cytotoxicity against K562 cells and contained many cytoplasmic granules, whereas the other population had low NK activity and a paucity of cytoplasmic granules.18 Further evidence that the granules may be involved in NK-mediated lysis derives from studies in patients with Chediak-
Higashi's syndrome, who also have impaired NK activity. In those patients, extremely large single cytoplasmic granules were evident in the cells that were otherwise morphologically LGL and reactive with HNK-1. Since FeR+ cells may mediate ADCC as well as NK, we studied two CLL patients' purified FeR+ cells for both NK and ADCC function and found both activities to be markedly impaired as compared to normal FeR+ cells (e.g., at effector-to-target ratio of 50:1, control ADCC was 28.7% ± 2.6% versus CLL ADCC of 3.1% ± 1.7%).

At least two hypotheses can be put forth to account for the marked deficiency in the intracytoplasmic azurophilic granules in the FeR+, OKM1+, and K562-binding cells isolated from the monocyte- and B-cell-depleted PBL of CLL patients. The first is that the NK cells may be involved in attempting to mediate destruction of an overwhelming number of malignant B cells present in the patients, and thus, the NK cells may be, in large part, degranulated when freshly isolated from the blood. In this regard, we and others have reported that CLL B cells are susceptible to lysis by NK cells. Alternatively, the agranular FeR+, OKM1+ cells in CLL patients may represent an immature cell in the NK lineage that has failed to mature. Although, at the present time, we have not distinguished between these possibilities, the impaired NK cell activity in patients with CLL, which may be related to the increased incidence of secondary malignancies in CLL, is probably due to the lack of cytoplasmic granules. These findings provide further evidence to support the contention that the granules present in normal individuals' NK cells may play a role in the cytolytic step of NK cells.

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