Patients With a Deficiency of Natural Killer Cell Activity Lack the VEP13-Positive Lymphocyte Subpopulation


Many patients with B-type chronic lymphocytic leukemia (CLL) exhibit a profound defect in their natural killer (NK) cell activity, the basis of which is still obscure. Hence, we analyzed the NK cells from peripheral blood samples from 11 patients with CLL for phenotype and function, after removal of the leukemic cells with a monoclonal antibody (BA-1) plus complement. Phenotypic analysis of these non-leukemic cells with monoclonal antibodies (MoAbs) against NK cells revealed that the CLL patients had higher percentages of HNK-1-positive cells (23.5% compared to controls with 14.7%). In contrast, VEP13-positive cells were absent or low in seven patients (0.8% compared to controls with 11.2%) and normal in four patients (10.5%). When testing NK cell activities against K562 or MOLT 4 target cells, patients with no or minimal numbers of VEP13-positive cells were found to be deficient, while patients with normal percentages of VEP13-positive cells had NK cell activity comparable to controls. Isolation by fluorescence-activated cell sorter of HNK-1-positive cells from patients lacking VEP13-positive cells and NK cell activity indicated that the majority of the HNK-1-positive cells in these patients had the large granular lymphocyte morphology that is characteristic of NK cells. Thus, the deficiency of NK cell activity in CLL patients appears to result from the absence of cells carrying the VEP13 marker.

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DEFICIENCIES of the immune system can be useful in the analysis of immune function, as the sequelae of a defined defect can give a clue to the understanding of the normal interaction of lymphocytes. In a previous report, we described the complete deficiency of natural killer (NK) cell activity in peripheral blood of patients with chronic lymphocytic leukemia (CLL), as detected after removal of the leukemic cells. Platooucsac et al. obtained similar results by studying purified T cells in such patients. The basis of this defect, however, remained obscure.

Recently, monoclonal antibodies (MoAbs) specifically reacting with human NK cells have become available. Two of these, designated HNK-1 and VEP13, were reported to define populations that contain the bulk of NK cell activity, as evidenced by complement (C) dependent lysis and fluorescence-activated cell sorter (FACS) separation. A detailed comparison of these two MoAbs revealed that the two large granular lymphocyte populations defined by HNK-1 and VEP13 do overlap, but are not identical. Functionally, HNK-1-positive lymphocytes contain a portion of NK cell activity and VEP13-positive lymphocytes contain almost all the NK cell activity in peripheral blood.

We report the use of the above MoAbs in the analysis of the deficiency of NK cell activity in patients with CLL, and we demonstrate that patients deficient in NK cell activity are also deficient in VEP13-positive lymphocytes, suggesting that the absence of lymphocytes expressing this surface marker is responsible for the deficiency of NK cell activity.

MATERIALS AND METHODS

Cell Isolation

Mononuclear cells from heparinized blood obtained from previously untreated consecutive B-CLL patients or from healthy controls were isolated by density gradient separation with Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) according to routine procedures. Diagnosis was based on clinical presentation and on marker analysis revealing monoclonality with respect to light chains. Aliquots of cells were frozen in the presence of 10% dimethyl sulfoxide and 20% heat-inactivated fetal calf serum (FCS) with a PTC 200 machine (Planer Ltd., Sunberry-on-Thames, UK) and then stored in liquid nitrogen. Previous experiments had shown that these procedures do not result in a change of lymphocyte phenotype and in only minimal decrease of NK cell activity. Directly before use, mononuclear cells were rapidly thawed in a 37°C water bath and washed twice.

Complement-Dependent Lysis With MoAb Against NK Cells

Mononuclear cells from healthy controls were treated with saturating amounts of the MoAb in the presence of rabbit C (Cedarlane, Hornby, Ontario, Canada) diluted 1:10 with RPMI 1640, 10% FCS (complete medium). After incubation for one hour at 37°C, the percent dead cells was determined by trypan blue dye exclusion, and the cells were washed three times in complete medium.

Complement-Dependent Removal of CLL B Cells

Cell populations were adjusted to 25 to 100 x 10^6/mL in RPMI 1640 with 10% FCS. One milliliter of cell suspension, 1 mL BA-1 mononuclear antibody (mouse ascites diluted 1:1,000), and 1 mL of

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undiluted rabbit complement (low-tox rabbit-complement H, Cedarlane) were mixed and the suspensions were incubated at 37 °C for one hour with repeated mixing in No. 2095 plastic tubes (Falcon, Oxnard, Calif.). The complement had been selected on the basis of high activity and absence of any detrimental effect on NK cell activity. After taking an aliquot for dead cell count with trypan blue dye exclusion, the suspensions were underlayered with Ficoll-Hypaque and spun at 800 g for 20 minutes. Cells from the interface were recovered and washed three times.

**Indirect Immunofluorescence**

For indirect immunofluorescence (IF) cells were first incubated for one-half hour at 4 °C with OKT-11, which is specific for T cells (Ortho Diagnostics, Heidelberg, FRG), 63D3, detecting the majority of monocytes (derived from the respective hybridoma obtained from ATCC, Rockville, Md), BA-1, recognizing B cells and granulocytes, HNK-1 or VEP13-biotin conjugate at saturating concentrations in phosphate-buffered saline, 2.5% FCS, and 0.02% NaN3 (IF buffer). After three washes, cells were incubated for another one-half hour with either goat anti-mouse IgG-FITC conjugate diluted 1:50 (Tago, Burlingame, Calif) or avidin-rhodamine diluted 1:50 (Vector, Burlingame, Calif), all in IF buffer. After three final washes, at least 200 cells were counted under a Leitz Orthoplan Fluorescence Microscope with Polomax epi-illumination. For cell sorting of HNK-1-positive cells, a FACS II (Becton Dickinson, Mountain View, Calif) was used with 488-nm exciting wavelength and photomultiplier tube at 650 to 800 V. Purities were greater than 95%.

**Killer Cell Assay**

Effector cells were adjusted to 2.5 to 5.0 × 10⁶ cells/mL, and cytotoxic activity was determined in a ⁵¹Cr release assay. For this purpose, the target cells, MOLT 4 and K562, were kept in continuous culture in RPMI 1640 with 10% FCS and were labeled at 37 °C with 100 μCi Na₂⁵¹CrO₄ (New England Nuclear, Dreieich, FRG) for one hour, washed four times, and adjusted to 5 × 10⁴/mL. Effector cells (100 μL) were titrated in triplicate in serial twofold dilutions in round-bottom microtiter plates (Costar, Cambridge, Mass). To each well, 100 μL target cells was added, resulting in effector to target ratios (E:T) of 100:1 to 50:1 and dilutions thereof. Plates were spun for two minutes at 50 g and incubated at 37 °C for six hours. One hundred microliter supernatant aliquots were taken and counted in a gamma counter. From these values, from the spontaneous release (target cells in medium alone), and from the maximum release (activity in 2.5 × 10⁴ target cells), the specific release was calculated using the formula:

\[
\text{specific release} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100.
\]

Lytic units (LU), with 1 LU defined as the effector cell number required to give a release of 15%, were calculated with a curve-fitting program using a Hewlett Packard HP67 calculator. The r values for all curves were greater than 95%.

**Pretreatment With β-Interferon**

Cell samples of 1 to 2 × 10⁶/mL in RPMI 1640, 10% FCS were incubated for one hour at 37 °C in the presence of 500 U/mL of fibroblast interferon (IFN; Fiblaferon, Rentschler, Laupheim, FRG), after which they were washed twice.

**Statistics**

For statistical evaluation, Student's t test was used.

**RESULTS**

**Effect of Anti-NK Cell MoAb Plus C on NK Cell Activity**

The two MoAbs, HNK-1⁴ (Leu-7, Becton Dickinson) and VEP13, both IgM antibodies derived from mouse ascites, were used together with selected rabbit C for treatment of lymphocytes from healthy donors. HNK-1 plus C depleted NK cell activity to a moderate extent, while after VEP13 plus C, almost all NK cell activity was removed, as depicted in a typical experiment (Fig 1) that is representative of altogether ten individuals tested. In terms of lytic units, with 1 LU defined as the number of effector cells required for 15% specific release, there is approximately a 50% reduction of cytotoxicity by HNK-1 plus C and a greater than 25-fold reduction by VEP13 plus C, since control treated cells contained 51.2 LU/10⁶ cells. HNK-1 and C treated cells contained 26.9 LU/10⁶ cells, while after VEP13 plus C, lytic units per 10⁶ cells were less than 2. The average percentage of cells killed by antibody and C in ten experiments was 10.9% ± 2.5% for HNK-1 plus C and 16.1% ± 8.4% for VEP13 plus C. Indirect immunofluorescence controls of the efficiency of depletion of the subpopulations in these experiments indicated that both HNK-1-positive and VEP13-positive cells were not detectable after treatment with the respective monoclonal antibodies and C. Thus, in the present study, HNK-1 appears to define about 50% of the active NK cells, while VEP13 recognizes almost all.

**Distribution of HNK-1-Positive and VEP13-Positive Cells**

A deficiency of peripheral blood lymphocyte function can either result from the absence of the relevant population or from a functional incompetence of the relevant lymphocytes present in normal numbers. This...
is exemplified by two types of severe combined immunodeficiency with antibody deficiency, where one type lacks B cells, while another has B cells, which, however, do not secrete appreciable immunoglobulin. In order to test these possibilities in a deficiency of NK cell activity, we assayed leukemia cell-depleted mononuclear cells from patients with CLL for the presence of HNK-1-positive and VEP13-positive lymphocytes.

For the study of nonleukemic cells in CLL we have established a highly efficient procedure for removal of the CLL leukemia cells using C-dependent depletion with the BA-1 monoclonal antibody. This procedure in CLL samples kills about 90% of the mononuclear cells (cf., Table 1), and after removal of dead cells by Ficoll-Hypaque separation, results in cell populations almost devoid of CLL cells (1.9% BA-1-positive cells) and with normal percentages of monocytes (23.1% 63D3-positive cells) and of T cells (69.6% OKT-11-positive cells). Cell populations, including the controls, were all subjected to the same BA-1 and C-depletion procedure. The average number of cells recovered after this procedure in the present study was $1.1 \pm 0.3 \times 10^6$/mL blood for controls and $1.0 \pm 0.6 \times 10^6$/mL blood for CLL. The analysis of such BA-1 plus C-purified cells by indirect immunofluorescence for MoAb-defined lymphocyte phenotype and in a $^{51}$Cr release assay for NK cell activity was performed in eight experiments (Table 1). Every experiment includes at least one CLL patient and one control (cf., Table 1, column 1). In Table 1, column 5, the high

<table>
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<th>Table 1. Analysis of Nonleukemic Lymphocytes From Chronic Lymphocytic Leukemia Patients for Phenotype and Function of Natural Killer Cells</th>
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*All subjects studied were males, except for one female control and one female patient, and all subjects were over the age of 52 years.†Stages according to K.R. Rai, A. Sawitsky, E.P. Cronkite, A.D. Chanan, R.N. Levy, and B.S. Pasternack: Blood 46:219–234, 1975.‡Not determined.§Percent dead cells was determined by trypan blue dye exclusion.¶Natural killer cell activity was determined using K562 or MOLT 4 target cells. Given are representative values of E:T ratios taken from the linear portions of the titration curves. The E:T's were identical for all donors in a given experiment.**Values for lymphocyte subsets are not given because individuals had been studied already in experiments 3 and 1, respectively, with results found to be similar. The values were omitted to allow for statistical analysis by Student's t test.††Difference not significant ($P > .05$) when compared to control group (Student's t test). The combined values for HNK-1 of both CLL groups, however, with a mean of 23.5% HNK-1-positive cells, were significantly higher compared to controls ($P < .05$).‡‡Difference significant ($P < .001$) when compared to control group.
killing efficiency for CLL cells of BA-1 and C is demonstrated, and in column 6, the high purity of the resultant populations with percentages of OKT-11–positive cells similar to normal controls. VEP13-positive cells, ranging from 7% to 15% were detected in the controls and in four of the CLL patients (Table 1, group I). The remaining seven patients had none or only few VEP13 cells (Table 1, group II). Compared to the control group, this difference was significant, with \( P < .001 \). Analysis with the HNK-1 monoclonal antibody revealed that all individuals studied had cells of this phenotype in peripheral blood. CLL patients of groups I and II had a significantly higher percentage of HNK-1 cells when compared to healthy controls (23.5% compared to 14.7%, \( P < .005 \)).

**Killer Cell Activities**

NK cell activity v leukemia cell targets (Table 1) was found to be unimpaired compared to controls in four patients with 8% to 15% VEP13-positive cells (group I). By contrast, NK cell activity was deficient in the seven patients with no or few VEP13-positive cells (group II). These findings were independent of the type of target cell used (MOLT 4 or K562). In Table 1, experiment 3, for instance, control donor Le, with 13.3% VEP13-positive cells, achieves a specific release from K562 of 26.3% at 25:1 E:T ratio. CLL patient Ste (group I), with 8.1% VEP13-positive cells, achieved 21.8% specific release. CLL patient Sc (group II), without detectable VEP13-positive cells, exhibits virtually no lysis (2.6%) under the same conditions in the same experiment. These findings were essentially identical in all other experiments shown. Deficiency of NK cell activity and of VEP13 cells was also found to be deficient (data not shown). Thus, some of the CLL patients are defective in their NK and K cell activity, and this deficiency appears to accompany the absence of VEP13-positive lymphocytes.

**FACS Separation of HNK-1 Cells**

We next questioned whether or not the HNK-1–positive lymphocytes found in the VEP13-deficient patients, based on large granular lymphocyte (LGL) morphology, can be assigned to the NK cell lineage. BA-1 plus C-depleted populations from patients Sch and Ma were stained for HNK-1 by indirect immunofluorescence and positive cells were isolated with the FACS. Pappenheim stains of cytopsin preparations showed 88% and 68% cells with the morphology of LGLs, ie, medium-sized lymphocytes with azurophilic granules, while the HNK-1–negative cells consisted of monocytes and small-sized lymphocytes without granules.

**Effect of Interferon on VEP13-Positive Cells and on NK Cell Activity in CLL**

We next asked whether or not \( \beta \)-IFN treatment of lymphocytes is able to increase the NK cell activity in CLL patients and if it will change the percentage of VEP13-positive cells. Data compiled in Table 2 demonstrate that \( \beta \)-IFN pretreatment will increase NK cell activity in controls but not or only minimally in CLL patients who are deficient for NK cell activity. The percentage of VEP13-positive cells does not change in controls, where NK cell activity is enhanced, or in CLL patients (Table 2); also, HNK-1–positive cells do not change (not shown).

**DISCUSSION**

The two monoclonal antibodies, HNK-1 and VEP13, both recognize cell populations with LGL morphology. Still, they appear to recognize different subsets of the NK cell lineage, since in our present study, HNK-1 plus C removes approximately half of the active NK cells, while VEP13 plus C removes almost all active NK cells. In initial reports, HNK-1 was found to recognize a larger portion of the active NK cells, while VEP13 removes almost all active NK cells, which was probably due to individual differences in antigen expression. In a model recently proposed, HNK-1 is expressed earlier within the NK cell lineage and is absent from the most mature cells.

The different specificities of HNK-1 and VEP13 prompted us to use them for analysis in CLL patients, where we and others reported a profound deficiency of

**Table 2. Effect of \( \beta \)-Interferon on VEP13-Positive Cells and on Natural Killer Cell Activity in CLL**

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<tr>
<th>Experiment</th>
<th>Subjects</th>
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*Mononuclear cells from CLL patients and controls were subjected to BA-1 and C treatment, followed by incubation for one hour with \( \beta \)-interferon (500 U/mL).
†The target cell was always K562.
NK cell activity. Because the CLL B cells, with their excessive numbers in many patients, can interfere with the NK cell assay, we removed them with a procedure that uses the mouse IgM antibody BA-1 plus C. Using this purification step, the phenotypic analysis of nonleukemic cells in CLL revealed an increase of HNK-1-positive cells compared to controls. Furthermore, in many patients, no or minimal numbers of VEP13-positive cells were found. The absence of the VEP13-defined antigen in some patients cannot be simply explained by a somewhat decreased antigen density, since we used high concentrations (100 μg/mL) of antibody in conjunction with the biotin-avidin system. Still, it is possible that the VEP13-defined antigen is expressed in very low amounts that are beyond the sensitivity of the method.

When looking at NK cell activities of the nonleukemic cells obtained from the CLL patients, we found samples with no or minimal VEP13-positive cells to be deficient, whereas samples with normal percentages of VEP13-positive cells exhibited levels of NK cell activity comparable to controls. These findings suggest that the absence of VEP13-positive cells is responsible for the deficiency of NK cell activity. However, this does not exclude that cells of the NK cell lineage without killer cell activity are present in patients lacking VEP13 cells. In fact, the HNK-1-positive cells, found to be expanded in the CLL patients, might represent such cells, and the LGL morphology of HNK-1-positive cells from patients lacking VEP13-positive cells supports this notion. Furthermore, the earlier observation of target-binding cells, which bind to NK cell-sensitive target cells, also suggests that cells of the NK cell lineage are present in CLL patients deficient for NK cell activity. In vitro pretreatment with β-IFN could not overcome the deficiency of NK cell activity in CLL. This is consistent with earlier studies in which no enhancement was seen in several patients after a similar procedure. In addition, the nonleukemic cells from patients treated with IFN did not increase beyond background levels after IFN pretreatment. Longer incubations with β-IFN or other types of IFN, however, might be effective.

In addition to VEP13, four other MoAbs that also recognize LGL and granulocytes have been described. In a comparative study, it could be demonstrated that they all react with epitopes on an Fc receptor specific to these cells. T lymphocytes, defined by rosetting with sheep red blood cells, contain a portion of NK cell activity that resides in their Fc receptor-positive fraction, and a portion of T cells from controls stain for VEP13. Many CLL patients exhibit increased percentages of Fc receptor-positive T cells. Our current results suggest that these expanded cells carry a type of Fc receptor different from the one specific for LGL and granulocytes, as many CLL patients are deficient in VEP13-positive cells.

Our finding of increased NK cell activity in healthy controls, without concomitant increase in VEP13-positive cells, indicates that IFN acts on VEP13-positive cells already present. Furthermore, in the absence of or with only low numbers of VEP13-positive cells, in group II CLL patients in this study, IFN is unable to induce NK cell activity.

The evidence presented here could be interpreted such that the expanded HNK-1-positive cells are precursor cells, blocked in differentiation toward active NK cells. Another possibility is that the HNK-1-positive cells act as suppressor cells and prevent the development of active NK cells carrying the VEP13 marker.

It is very important to understand these mechanisms since, as suggested earlier, the profound deficiency of NK cell activity in CLL might be responsible for the high rate of secondary malignancies and infections.

ACKNOWLEDGMENT

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