Lysis of Lymphoma Cells by Autologous and Allogeneic Natural Killer Cells

By Kazuo Oshimi, Yoko Oshimi, Osamu Yamada, and Hideaki Mizoguchi

Studies were undertaken to determine whether natural killer (NK) cells would lyse autologous and allogeneic lymphoma cells. When large granular lymphocytes, which are known to mediate NK activity, were enriched from peripheral blood and used as effector cells, they lysed autologous lymphoma cells of all of eight patients tested, and those of healthy donors lysed lymphoma cells of all of ten patients tested. The addition of interferon to the culture medium enhanced their cytotoxicity in three of the eight patients in the autologous effector-tumor system and in four of the ten patients in the above allogeneic system. On the basis of the unlabeled target competition test and the decrease in cytotoxicity with anti-NK antibody treatment, NK cells appeared to be the main cytotoxic effector cells for autologous and allogeneic lymphoma cells.

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MATERIALS AND METHODS

Characteristics of Patients and Tumor Cells

As shown in Table 1, ten patients with non-Hodgkin's lymphoma were studied. The diagnosis of lymphoma was based on the Working Formulation. The percentage of malignant cells in the specimens was determined from morphology and surface markers. For the analysis of surface markers, lymph nodes or other tissues surgically obtained were assayed as described elsewhere. Patients 1, 2, 3, 4, 5, 7, and 8 were treated with combination chemotherapy CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisolone), and patient No. 6 had radiotherapy alone. Because all the patients studied belonged to intermediate to unfavorable prognostic groups, the chemotherapy given consisted of aggressive regimens, including doxorubicin.

Preparation of LGL-Rich Lymphocytes

The preparation of LGL-rich lymphocytes was described previously. Heparinized whole blood was obtained from the patients and healthy donors. Patient's blood was taken at the periods when lymphoma cells were not present in the peripheral blood. To minimize the effects of chemotherapy and radiotherapy, blood was obtained before the initiation of therapy (patients No. 6, 7, 9) or at least three weeks after the cessation of therapy (patients No. 2, 3, 5, 8, 10). PBMCs were separated with Ficoll-Conray density gradients, washed twice, and suspended in RPMI 1640 medium containing 10% fetal calf serum (FCS). The cells were then applied to columns of nylon wool and incubated for one hour at 37°C. Nonadherent
cells were eluted with warm medium and were added to Percoll density gradient solutions. To prepare density gradients, Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) was autoclaved and mixed in various concentrations with RPMI 1640 containing 10% FCS. The Percoll solutions in volumes of 1.5 mL were carefully layered in a 10-mL round-bottom test tube starting with 40% Percoll (fraction 5) and graded by 2.5% concentration reductions to 30% Percoll on the top (fraction 0). After the lymphocytes were added to the Percoll solutions, they were centrifuged at 650 g for 20 minutes at room temperature. Each fraction was collected, washed twice, and then low-density fractions 1 and 2 were mixed and used as LGL-rich lymphocytes. The LGL-rich lymphocytes were then incubated for 24 hours with and without 1,000 or 3,500 IU of interferon-β (IFN-β)/mL and used as effector cells for the cytotoxicity assay. IFN-β was induced in human fibroblasts by the addition of polyinosinic-polycytidylic acid and had a specific activity of 2 × 10^11 U/mg of protein.31 First, we used a 1,000 U/mL concentration of IFN and later changed to 3,500 U/mL because cytotoxic activity against lymphoma cells was not enhanced at 1,000 U/mL and because IFN at 3,500 U/mL, but not at 500 U/mL, significantly enhanced the cytotoxicity of whole PBMCs for autologous lymphoma cells in the previous study.32 The percentage of LGLs in LGL-rich lymphocytes was determined by morphological examination of May-Grünwald-Giemsa–stained cytocentrifuged smears. High-density fractions 3 and 4 or a mixture of these fractions were also used as effector cells for the cytotoxicity assay in the same way.

**Preparation of Target Cells**

K562 cells were used as target cells for NK assay. They were maintained in RPMI 1640 medium supplemented with 10% FCS. Teased lymph node cells were suspended in Eagle’s minimum essential medium containing 75% FCS and 10% dimethylsulfoxide and stored in liquid nitrogen. When used as target cells, the cells were thawed and their viability was determined by means of the trypan blue dye exclusion method. Cells of more than 70% viability were used as target cells.

For the labeling of target cells in the cytotoxicity assay, 50 μCi or 300 to 400 μCi of Na$_2$CrO$_4$ (Japan Isotope Association, Tokyo) was incubated with 10^6 cells in 0.2 mL of RPMI 1640 with 10% FCS for two hours, then washed four times, and used as target cells. Fifty microliters were used for the labeling of cultured K562 cells and 300 to 400 μCi for the lymphoma cells isolated from the patients. In preliminary experiments, the susceptibility to NK cells of frozen K562 cells used immediately after thawing did not differ from that of K562 cells in culture. Therefore, the lymphoma cells isolated from the patients were used immediately after thawing.

**Assay for Cytotoxic Activity**

A $^{51}$Cr release test was used for the assay of cytotoxic activity. $^{51}$Cr-labeled target cells, 8 × 10^3 in 50 μL of RPMI 1640 containing 10% FCS, were added to each well of effector cells, and the plates were further incubated for five hours. Effector-target ratios (E:T) were 40:1 and 10:1 when lymphoma cells were used as target cells, and 10:1 and 2:1 when K562 cells were used as target cells. The experiments were performed in triplicate. At the termination of the $^{51}$Cr-release test, the supernatant fluid was harvested with the Titer tek supernatant collection system (Flow Laboratories, Rockville, Md), and the radioactivity was determined in a gamma well counter. The specific $^{51}$Cr release was calculated by the following formula: % release = [(experimental $^{51}$Cr release – spontaneous $^{51}$Cr release) / (maximum $^{51}$Cr release – spontaneous $^{51}$Cr release)] × 100, where spontaneous release is the $^{51}$Cr release from 8 × 10^3 labeled target cells incubated alone in the medium in the presence or absence of IFN, and maximum release is that from labeled cells incubated alone in water containing 5% detergent. Tests in which spontaneous release exceeded 60% of maximum release were excluded from the results. Spontaneous release in the presence of IFN did not differ from that in its absence, indicating that IFN was not directly cytoxic to the target cells during a five-hour $^{51}$Cr-release assay. The results of cytotoxicity were expressed as mean percent specific $^{51}$Cr release. The standard deviation of percent specific $^{51}$Cr release was less than 10% in all experiments. Cytotoxic activity was considered positive when the value of experimental $^{51}$Cr release was significantly above that of spontaneous $^{51}$Cr release by the Student’s t test.

**Characterization of Cytotoxic Effector Cells**

In order to determine whether the effector cells reactive with patients’ lymphoma cells were NK cells or not, the following methods were used. First, an unlabeled target competition test was performed.15,20 Before the addition of $^{51}$Cr-labeled lymphoma cells, graded numbers of unlabeled K562 cells or Raji cells were added to the LGL-rich effector cells. If killer cells reactive with patients’ lymphoma cells were included within the population reactive with K562 cells but not with Raji cells, unlabeled K562 cells, but not Raji cells, added during the $^{51}$Cr-release test would inhibit the lytic activity against $^{51}$Cr-labeled lymphoma cells. Because K562 cells are highly sensitive to NK cells and Raji cells are relatively resistant to them, effector cells that are strongly inhibited in their cytotoxicity by unlabeled K562 cells are considered to be NK cells. Second, it was determined whether cells depleted of NK cells by monoclonal antibody Leu-11b or of T cells by monoclonal antibody OKT3 had cytotoxic activity against lymphoma cells. The panning method described by Payne et al13 was modified as follows. Three-milliliter quantities of affinity-purified F(ab')2 fragment goat anti-mouse IgG or IgM (Cappel Laboratories, West Chester, Pa) were added to 60-mm Petri dishes (Falcon, Oxnard, Calif). After overnight incubation at 4 °C, the antibody was harvested from the plates and each plate was washed three times with 3 mL of cold medium. LGL-rich lymphocytes, 10^5, resuspended in 1 mL of 1:10-diluted Leu-11b or
OKT3 monoclonal antibody were incubated on ice for 30 minutes, washed, resuspended in 2.5 mL of RPMI 1640 containing 5% FCS, and added to the antibody-coated plates. Because Leu-11b and OKT3 are IgM and IgG antibody, respectively, Leu-11b-treated cells were added to anti-mouse IgM antibody-coated plates and OKT3-treated cells were added to anti-mouse IgG antibody-coated plates. The plates were then incubated in a 3% CO2 incubator at 37°C for 30 minutes. The nonadherent cells (Leu-11b-negative or OKT3-negative cells) were recovered by swirling the plates and removing the cells by aspiration with a Pasteur pipette. The cells were centrifuged, and the pellet was resuspended in RPMI 1640 containing 10% FCS. The concentration of cells was readjusted, and the cells were used as effector cells for the cytotoxicity assay. In preliminary experiments, more than 95% of the Leu-11b-positive or OKT3-positive cells were removed by the panning method when the cells were assayed by the indirect immunofluorescence method.

RESULTS

Sensitivity of Lymphoma Cells to Autologous and Allogeneic LGL-Rich Effector Cells

As shown in Table 2, lymphoma cells of all of the eight patients tested were sensitive to autologous LGL-rich effector cells, ie, effector cells from a mixture of fractions 1 and 2. Moreover, the lytic activity in three patients was significantly enhanced by the addition of IFN at an E:T of 40:1 or 1:10. The sensitivity did not differ with a difference in histology or surface markers of the lymphoma cells, and the percentage of LGLs in effecton cells did not correlate with the lytic activity. NK activity against K562 cells varied from patient to patient and did not correlate with the percentage of LGLs. NK activity was augmented in all the experiments by the addition of IFN at an E:T of 1:10:1 or 2:1 or both. Because the level of cytotoxicity did not differ significantly between the patients whose blood was taken before therapy and those whose blood was taken after the cessation of therapy, it seemed unlikely that the treatment profoundly affected the cytotoxic activity for lymphoma cells or K562 cells when blood was taken at least three weeks after the cessation of therapy. The cytotoxic activity of LGL-depleted fractions, ie, fractions 3 and 4, or a mixture of these fractions, was always lower than that of LGL-rich fractions against lymphoma cells and K562 cells (data not shown).

LGL-rich effector cells of one or both of two healthy donors killed allogeneic lymphoma cells of all ten patients, and the treatment with IFN augmented their cytotoxicity in four patients (Table 3). In five repeated experiments, the percentage of LGLs in the LGL-rich lymphocytes of donor 1 was 37% to 56% (mean, 47%) and their NK activity against K562 cells was 22% to 80%.

Table 2. Sensitivity of Lymphoma Cells to Autologous LGL-Rich Effector Cells

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Percentage of LGLs*</th>
<th>IFN in Culture (U/mL)</th>
<th>Target: Lymphoma Cells</th>
<th>Target: K562 Cells</th>
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<td>3,500</td>
<td>37.4††</td>
<td>24.9††</td>
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LGL-rich lymphocytes obtained from the patients were incubated for 24 hours with and without 1,000 or 3,500 units of IFN/ml, and their cytotoxicity was assayed against autologous lymphoma cells and K562 cells by a five-hour 51Cr-release test at E:T of 40:1, 10:1, and 2:1.

*The percentage of LGLs in LGL-rich effector cells was determined with May-Grunwald-Giemsa-stained smears.
†Percentage of cytotoxicity in a 51Cr release assay in which the value of released 51Cr was significantly (P < .05) higher than that of spontaneously released 51Cr in an autologous effector-tumor system.
‡Percentage of cytotoxicity significantly (P < .05) enhanced by the addition of IFN as compared with control cytotoxicity in an autologous system.

Table 3. Sensitivity of Lymphoma Cells to Healthy Donors' LGL-Rich Effector Cells

<table>
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<tr>
<th>Patient No.</th>
<th>IFN in Culture (U/mL)</th>
<th>Target: Lymphoma Cells</th>
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<th>E:T 10:1</th>
<th>E:T 40:1</th>
<th>E:T 10:1</th>
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LGL-rich lymphocytes from healthy donors 1 and 2 were incubated for 24 hours with and without 1,000 or 3,500 units of IFN/ml, and their cytotoxicity was assayed against lymphoma cells isolated from the patients.

*Values of significant cytotoxicity (P < .05). See footnotes † and ‡, respectively, of Table 2.
44% (mean, 32%) at an E:T of 10:1 and 4% to 12% (8%) at 2:1, whereas in donor 2, LGLs constituted 46% to 66% (57%) and their NK activity was 38% to 67% (52%) at 10:1 and 9% to 25% (17%) at 2:1. In each experiment, NK activity of both donors was significantly enhanced by the addition of IFN at 1,000 and 3,500 U/mL (data not shown).

Characterization of Cytotoxic Effector Cells

In order to see whether the effector cells reactive with lymphoma cells were NK cells or not, LGL-rich lymphocytes from patients No. 6 and 10 and from healthy donors 1 and 2 were mixed with 51Cr-unlabeled K562 cells or Raji cells before the addition of 51Cr-labeled lymphoma cells in competitive inhibition assays. The results depicted in Fig 1 show that unlabeled K562 cells strongly inhibited the killing of lymphoma cells, whereas unlabeled Raji cells inhibited the killing of these target cells at higher inhibitor-target ratios, suggesting that killer cells reactive with lymphoma cells were included within the population of killer cells reactive with K562 cells. LGL-rich lymphocytes from the other healthy donor (donor 2) showed the same pattern of inhibition (data not shown). In addition, effector cells from LGL-rich fractions showed higher cytotoxicity than those from LGL-depleted fractions against autologous and allogeneic lymphoma cells (data not shown).

To further investigate the nature of the effector cells, LGL-rich lymphocytes from patients No. 6 and 7 and from healthy donors 1 and 2 were treated with monoclonal antibody Leu-11b or OKT3, and NK cells or T cells were depleted by the panning method. The nonadherent Leu-11b-negative or OKT3-negative cells were tested for their cytotoxicity for autologous and allogeneic lymphoma cells. As shown in Table 4, treatment with Leu-11b antibody markedly inhibited the cytotoxicity for K562 cells, indicating that NK activity was almost totally eliminated by the removal of Leu-11b–positive cells. Likewise, the cytotoxicity for lymphoma cells was significantly inhibited by Leu-11b antibody treatment in both the autologous and the allogeneic systems. However, the inhibition was less pronounced than that of NK activity for K562 target cells. When the effector cells were depleted of OKT3-positive cells, the cytotoxicity for K562 cells was enhanced and the cytotoxicity for lymphoma cells was almost the same as or slightly less than the control cytotoxicity. These results suggest that mainly NK cells mediated cytotoxicity for lymphoma cells in the autologous and allogeneic systems. However, the possibility that effector cells other than NK cells, probably T cells, mediated part of the cytotoxicity for lymphoma cells but not for K562 cells cannot be ruled out.

DISCUSSION

It has been reported that freshly isolated tumor cells from cancer patients are relatively resistant to lysis by autologous blood lymphocytes in short-term assays. Our previous study demonstrated that lymphoma cells isolated from patients are also resistant to lysis by autologous PBMCs. In our present study, however, LGL-rich lymphocytes lysed autologous lymphoma cells in all of eight patients tested (Table 2). Therefore, it must be determined whether the latter positive results were obtained because of enrichment of effector cells by the Percoll gradient sedimentation method or because of the removal of suppressor cells during the preparation of LGLs. We recently observed that monocytes obtained from the peripheral blood of patients with lymphoma and of healthy individuals markedly inhibited the cytotoxicity of LGL-rich lymphocytes for autologous and allogeneic lymphoma cells, suggesting that suppressor monocytes are present in the peripheral blood of both patients and healthy individuals and that the removal of monocytes by passing PBMCs through nylon wool columns in the process of preparing LGLs unmasks the presence of killer cells in a 24-hour culture (manuscript in preparation).

IFN is known to enhance NK activity. In our present study, cytotoxicity for K562 cells was significantly enhanced by the addition of IFN in all the experiments, whereas cytotoxicity for autologous and allogeneic lymphoma cells was enhanced in less than
half the patients (Tables 2 and 3). Our failure to find consistent enhancement of lysis by IFN-treated effector cells agrees with similar reports.\textsuperscript{18,20,21} Although the reason for this failure remains to be clarified, it may be partly explained by the fact that suppressor monocytes were removed in the process of preparing LGLs. That is, the removal of monocytes unmasked the presence of killer cells in a 24-hour culture and the enhancement of cytotoxicity by this unmasking was so marked that the enhancement by IFN would be overshadowed.

Effector cells mediating cytotoxicity for autologous fresh tumor cells have been shown to be T cells by E-rosette formation.\textsuperscript{17} It cannot be ruled out, however, that NK cells are involved in lysis of autologous tumor cells, since NK cells have low-affinity E receptors.\textsuperscript{34} Indeed, Uchida and Micksche\textsuperscript{35} recently demonstrated that LGLs lysed autologous cancer cells in 15 of 22 patients. Serrate et al\textsuperscript{19} reported similar results in one patient with solid tumors. However, these reports did not mention a study of surface markers of the effector cells. Our present study demonstrated that Leu-11b-positive NK cells were the main effector cells for autologous and allogeneic lymphoma cells (Table 4). It is interesting to note, however, that compared with the effector cells cytotoxic for K562 target cells, the effector cells for lymphoma target cells were less efficiently eliminated by the depletion of NK cells with Leu-11b monoclonal antibody in autologous and allogeneic systems. This could be explained if OKT3-positive T cells mediated part of the cytotoxicity for lymphoma cells but not for K562 cells. This presumption is based on the finding that NK cells lytic for K562 cells were enriched by the removal of OKT3-positive cells, whereas the effector cells lytic for lymphoma cells were not enriched, and were even depleted in some experiments, by the same treatment. It is possible that killer T cells specific for autologous lymphoma cells were present in the patients’ PBMCs and were enriched in low-density LGL-rich fractions. It remains to be clarified, however, whether killer T cells other than NK cells that are reactive with lymphoma cells are present in healthy donors’ PBMCs or are induced in vitro in a short-term culture.

In this article, we demonstrated that NK cells lytic for autologous lymphoma cells might be present in the peripheral blood of patients. If these NK cells act in vivo, why does the lymphoma develop and spread? This might be explained by the presumption either that the results obtained from the experiments represent only an in vitro phenomenon, or that, although NK cells lyse lymphoma cells in vivo, the lytic activity is not strong enough to prevent the development of lymphoma, or it is inhibited by suppressor cells.

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REFERENCES

3. Saksela E, Timonen T, Ranki A, Häyry P: Morphological and
LYMPHOMA CELL LYSIS BY NK CELLS

functional characterization of isolated effector cells responsible for human natural killer activity to fetal fibroblasts and to cultured cell line targets. Immunol Rev 44:71, 1979


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