A Killing Defect of Natural Killer Cells With the Absence of Natural Killer Cytotoxic Factors in a Child With Hodgkin’s Disease

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A killing defect of natural killer (NK) cells in the absence of NK cytotoxic factors (NKCF) was first demonstrated in a child with Hodgkin’s disease. The patient lacked detectable NK cell activity in every phase of the disease as measured by a four-hour ⁵¹Cr-release assay using K562 cells as a target. The percent lysis at a 40:1 effector:target ratio by the patient’s lymphocytes was persistently below 0.3% as compared with the normal lymphocyte value of 46.2% ± 5.8% (mean ± SD). NK cell activity was not detectable at effector:target ratios of 10:1 to 80:1 and by prolongation of the incubation time, and the NK cell defect was not restored or improved by lymphocyte stimulation with polyinosinic-polycytidylic acid, interferon (IFN)-α, or interleukin 2 (IL-2). The numbers of Leu-7+ cells and Leu-11+ cells were normal as counted by flow cytometry. A single cell-in-agarose assay demonstrated normal numbers of target binding cells (TBCs), and they showed the morphology of “large granular lymphocytes.” However, there were no TBCs with dead targets. These results indicated that the patient’s lymphocytes contained normal numbers of NK cells that were capable of recognizing and binding to a target but were incapable of killing the bound target cell. The patient’s lymphocytes were then studied for their release of NKCF upon interaction with K562 cells. The patient’s cells did not release NKCF, and the NK cell defect was not restored or improved by stimulation of the cells with IFN or IL-2. It is suggested that the deficient release of NKCF may have been related to the killing defect of the NK cells in this patient.

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Natural Killer (NK) cells are considered to be non-T, non-B lymphocytes and play important roles in the defense against viral infections and tumor growth. NK cells can lyse a target cell through recognition, binding, killing, and detaching, and they repeat this lytic sequence by the recycling capacity. Soluble NK cytotoxic factors (NKCF) and NK granule cytolytic, which are released from NK cells after contact with NK-sensitive target cells, are known to be involved in the killing step.

A variety of functional defects have been documented in such leukocytes as neutrophils, monocytes/macrophages, T cells, and B cells, but little is known about dysfunctions of NK cells. Targan and Oseas demonstrated a NK cell defect in Chédiak-Higashi syndrome. In this syndrome, a relative refractoriness in initiating the postbinding lytic mechanism leads to the lack of NK cell activity, which was termed lazy NK cells. We have recently demonstrated that there is impaired recycling of NK cells as a cause of depressed NK cell activity in some types of childhood chronic neutropenia.

We studied a child with Hodgkin’s disease who lacked detectable levels of NK cell activity for more than 4 years, and we analyzed the NK cell defect. In this article, we demonstrate a killing defect of NK cells in the absence of NKCF.

Case Report

A 7-year-old boy presented in January 1982 with cervical lymphadenopathy. He had suffered from recurrent infections such as upper respiratory tract infections and otitis media since infancy. The family history was unremarkable.

At presentation, there were two enlarged lymph nodes measuring 2 cm x 2 cm x 2.5 cm in the left and in the right cervical region. The hemoglobin level was 13.8 g/dL; the platelet count 379 x 10⁵/μL; and the leukocyte count 7,400/μL with 37% neutrophils, 3% eosinophils, 8% monocytes, and 52% lymphocytes. The bone marrow was normal. The serum IgM, IgG, and IgA levels were 57, 483, and 55 mg/dL, respectively. Delayed hypersensitivity against candidal antigen was positive. Liver and renal function test results were normal. Chest x-ray films, bipepal lymphangiography, echography, and computerized tomography did not show mediastinal and retroperitoneal lymphadenopathy. A barium scan demonstrated increased accumulation in the left cervical region. A specimen from excisional cervical lymph node biopsy revealed Hodgkin’s disease of a mixed cellularity type.

On March 9, the patient was started on the first of six cycles of MOPP, which consisted of 6 mg/m² nitrogen mustard intravenously (IV) on days 1 and 8, 1.4 mg/m² vincristine IV on days 1 and 8, 100 mg/m² procarbazine orally daily for 14 days, and 40 mg/m² prednisolone orally daily for 14 days. Two days later, irradiation (4,000 rad in total) to the bilateral cervical regions was initiated. The cervical lymphadenopathy disappeared within 2 weeks after the institution of the treatments. The patient completed the therapy by the end of August 1982, and he has been in complete remission for more than 4 years. He is doing well except for frequent episodes of upper respiratory tract infections.

Materials and Methods

Sample preparation. Heparinized peripheral blood samples were obtained from the patient before, during, and after therapy. Mononuclear cells were separated by Ficoll-Hypaque centrifugation from the blood samples, depleted of monocytes by adherence, and used as the patient’s lymphocytes. For comparison, lymphocytes were obtained from six children with Hodgkin’s disease ranging in age from 5 to 15 years; their clinical stages (An Arbor) were IA, IIA,
or IIb, and the histologic subtypes (Rye) were lymphocytic predominance in four and mixed cellularity in two of them. Normal lymphocytes were also obtained, in an identical manner, from healthy volunteers.

For some experiments, lymphocytes (2 to 3 x 10^6/mL) were incubated with 30 μg/mL polyinosinic-polyctydilic acid (poly I:C; Calbiocheh Behring, La Jolla, CA), 1,000 U/mL recombinant interferon (IFN)-α (Kyowa Hakko Co, Tokyo), or 1,000 U/mL recombinant interleukin 2 (IL-2) (Takeda Seiyaku Co, Osaka, Japan) at 37°C for 20 hours in a 5% CO2 atmosphere. They were washed and used as effector cells.

For testing the inhibition of NK cells by serum, serum samples were prepared from the patient and healthy volunteers.

**Surface marker analysis.** Percentages of lymphocytes bearing Leu-7 or Leu-11 were counted by flow cytometry using anti-Leu-7 and anti-Leu-11 monoclonal antibodies (Becton Dickinson, Sunnyvale, CA).

**31Cr release assay.** NK cell activity was assayed on 31Cr-labeled K562 cells as previously described. In some experiments, normal lymphocytes were incubated with the patient's serum or normal serum at 37°C for 30 minutes, suspended in culture medium with 20% of the serum, and used as effector cells. Cell mixtures, prepared at various effector:target ratios, were brought to a final volume and incubated at 37°C for 4, 8, 12, 16, and 24 hours in a CO2 atmosphere. The following equation was used to express cytotoxicity: percent lysis = ([cpm spontaneous release - cpm spontaneous release]/[cpm maximal release - cpm spontaneous release]) x 100. All assays were set up in triplicate.

Antibody-dependent cell-mediated cytotoxicity (ADCC) was similarly assayed by using a target of Raji cells coated with rabbit anti-Raji antibody.

**Single cell-in-agarose assay and morphological examination of target-binding cells.** This assay was done according to a modification of the method of Grimm and Bonavida as previously described. Effector lymphocytes (2 x 10^6 cells/mL) and unlabeled target K562 (2 x 10^6 cells/mL) were mixed, centrifuged at 500 g for two minutes, and incubated at 37°C for ten minutes. The cell mixture was added to 0.5 mL of 0.5% agarose in a liquid form. The cells and agarose were mixed, and 0.1 mL to 0.2 mL of the mixture was quickly poured onto culture plates that were precoated with 0.5% agarose. After the cell mixture in agarose had solidified, the culture medium was layered and incubated at 37°C for 4, 12, and 18 hours. After the incubation, the medium was removed, and 0.1% trypsin blue was added. Percentages of target-binding cells (TBCs) and TBCs with dead targets were determined under a microscope.

Part of the mixture of effector K562 cells was processed for morphological examination of TBCs. The samples were spun in a cytocentrifuge (Cytospin, Shandon Southern, Elliott, IL) and stained with May-Grünwald-Giems staining.

**Preparation of cell-free supernatant containing NKCF.** NKCF in a cell-free supernatant were prepared by the methods of Wright and Bonavida and Depligioni et al. The patient's and normal lymphocytes (5 x 10^6 cells/mL) were cultured at 37°C for 18 hours in the absence or presence of 1,000 U/mL recombinant IFN-α (Kyowa Hakko Co) or 1,000 U/mL recombinant IL-2 (Takeda Seiyaku Co). After the culture, the cells were washed and suspended at a concentration of 3 x 10^6 cells/mL in culture medium containing K5-62 cells (6 x 10^5 final concentration). The cell mixtures were cultured at 37°C for 4, 8, 16, and 24 hours. The cells were then centrifuged, and cell-free supernatants were harvested and processed for cytotoxicity assays.

**Assay for NKCF.** NKCF in the supernatants were measured by an 18-hour [31Cr-release assay using K562 cells as a target. Briefly, a 50% concentration of the supernatant was added to [31Cr-labeled K562 (5 x 10^5 cells/mL) in a 100-μL final volume in triplicate wells of microtiter plates. After an 18-hour incubation, 100 μL of phosphate-buffered saline was added to each well, and 100 μL of the supernatant was collected after spinning. Cytotoxicity was expressed by the percentage of lysis as described.

**RESULTS**

**Numbers of lymphocytes bearing Leu-7 or Leu-11.** The numbers of Leu-7' cells and Leu-11' cells were counted in the patient after termination of MOPP therapy. Percentages (mean ± SD) of Leu-7' cells and Leu-11' cells were 14% ± 2% and 10% ± 2%, which were normal compared with the normal lymphocyte values of 18% ± 8% and 15% ± 6%, respectively.

**Cell-mediated cytotoxicity as determined by [31Cr-release assay.** NK cell activity of the patient's lymphocytes was assayed once at the time of diagnosis, once during MOPP therapy, and on seven separate occasions during a 4-year period after termination of the therapy. In every assay, the patient's lymphocytes did not have detectable levels of NK cell activity against NK-sensitive K562 cells: the percent lysis was persistently below 0.3% at a 40:1 effector:target ratio, which was below 0.6% of the normal lymphocyte value of 46.2% ± 5.8% (mean ± SD). Representative results of NK cell activity assays performed in February 1986 are shown in Table 1.

For comparison, NK cell activity was assayed in six children with Hodgkin's disease at the time of diagnosis and/or during a 3-year period after termination of MOPP therapy or radiotherapy. The control patients' lymphocytes at diagnosis had detectable levels of NK cell activity, although the activity was depressed in two of the four patients studied: the percent lysis values in the two were 15.7% and 28.6%, which were below 2 SD of the normal lymphocyte value. The lymphocytes from six patients after termination of the therapy had normal levels of NK cell activity: the percent lysis ranged from 40.1% to 49.2% as compared with the normal lymphocyte value of 46.2% ± 5.8% (mean ± SD).

The patient's lymphocytes did not show NK cell activity at effector:target ratios of 10:1, 20:1, 40:1, and 80:1: the
percent lysis values were below 0.5% at all of the effector:target ratios.

Next, experiments were performed to examine whether the patient's NK cells could respond to in vitro poly I:C, IFN, or IL 2. The results are shown in Table 1. Stimulation of the patient's lymphocytes with poly I:C, IFN, or IL 2 did not significantly augment their cytotoxicity.

The kinetics of NK cell activity of the patient's lymphocytes were tested for cytolysis against K562 target cells at a 40:1 effector:target ratio at five time points over a 24-hour period. As depicted in Fig 1, the patient's lymphocytes did not show cytotoxicity at any time point of the assay. This result certainly precluded the possibility of their delayed killing as a cause of the lack of NK cell activity in the conventional four-hour assay. The responsiveness of the patient's lymphocytes to IFN was also studied under an identical condition. Stimulation of the patient's lymphocytes with IFN did not increase their cytotoxicity at any time point (Fig 1).

Experiments were performed to determine whether the patient's serum had inhibitory effects on normal NK cell activity. There was no difference in cytotoxicity between normal lymphocytes treated with the patient's serum and those treated with normal serum: the percent lysis by the former cells was 96.2% and that by the latter cells was 97.0% of the control value obtained without the serum treatment (Table 2). This result indicated that the lack of NK cell activity was not due to a serum inhibitory factor(s) but a cellular defect.

ADCC of the patient's lymphocytes was assayed against Raji cells that were coated with anti-Raji antibody when the patient was not receiving therapy. The patient's lymphocytes had normal levels of cytotoxicity against the target: the percent lysis (mean ± SD) was 21.4% ± 2.6% as compared with the normal lymphocyte value of 26.3% ± 3.6% at a 20:1 effector:target ratio.

**NK cell activity as determined by single cell-in-agarose assay and morphology of TBCs.** Because NK cells kill target cells after binding to them, experiments were performed after termination of MOPP therapy to determine whether the patient's lymphocytes contained NK cells capable of binding to K562 (TBCs) and, if present, whether they could kill them (TBCs with dead targets) at the single-cell level. In the patient's lymphocytes, the percentage of TBCs was normal: the value (mean ± SD) was 9.3% ± 2.0% as compared with the normal lymphocyte value of 8.3% ± 1.3%. There were no TBCs with dead targets, however, after incubation for 4, 12, and 18 hours in the patient's lymphocytes: the value was below 1% of TBCs as compared with the normal lymphocyte value of 25.6% ± 3.2% (mean ± SD) after a four-hour incubation.

Since NK cells are morphologically identifiable as large granular lymphocytes, 16 morphological observations were performed to examine whether TBCs had the morphology of NK cells. Most of the patient's TBCs as well as normal TBCs contained azurophilic granules in the abundant cytoplasm. Thus these results provided a line of evidence for an NK cell origin of the patient's TBCs that were present in a normal number and were capable of recognizing and binding to K562 cells but were incapable of killing them.

**NKCF release.** NKCF activity was measured by an 18-hour 51Cr-release assay on K562 cells. The results are shown in Fig 2. The patient's lymphocytes did not release detectable levels of NKCF activity at several time points of the culture over a 24-hour period, which indicated a deficiency of NKCF production by the patient's NK cells. The defect could not be improved by lymphocyte treatment with IFN or IL 2 in vitro.

**DISCUSSION**

Our patient is characteristic in persistently lacking NK cell activity in every phase of Hodgkin's disease, including the remission phase and while not receiving therapy. There was no restoration or improvement of the NK cell defect by lymphocyte treatment with poly I:C, IFN, or IL 2 in vitro. In this patient, we have demonstrated a killing defect of NK cells with the absence of NKCF.

When NK cells work as effector cells for cytolysis, they first recognize and bind to a target cell, 43 and then the NK cells kill the bound target by releasing lytic materials such as...
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NK granule cytolysis has a potent lytic activity against NK-sensitive target cells, and its activity is functionally different from that of NKCF in potency, target cell selectivity, kinetics of cytolysis, and calcium requirement.\(^7\) It would be interesting, therefore, to study NK granule cytolysis in our patient. Unfortunately, we could not study it in this patient because it was difficult to obtain sufficient numbers of NK cells (large granular lymphocytes). The total absence of NK cell activity indicates some abnormality of NK granule cytolysis in the patient's NK cells, although their cytoplasmic granules were morphologically normal, and further studies are expected to confirm this.

Our patient may have acquired the NK cell deficiency as one of the cell-mediated immune defects in Hodgkin's disease,\(^1\) but why he has such a persistent and profound immunodeficiency remains obscure. In some patients with Hodgkin's disease, NK cell activity is depressed before and/or during chemotherapy or radiotherapy,\(^8\) but becomes normal after attaining complete remission or after termination of therapy.\(^9\) Actually, some of our control patients with Hodgkin's disease had depressed but detectable levels of NK cell activity at diagnosis, and all of them had normal levels of NK cell activity after therapy. It is known that depression of NK cell activity is not related to the clinical stage or histological type of Hodgkin's disease,\(^10\) this was the same in our studies. Serum inhibitory factors are often described in this disease,\(^11\) but the possibility of such factors as a cause of the NK cell deficiency can be excluded by the absence of inhibitory effects of the patient's serum on the normal NK cell function, as demonstrated here. This result further indicates that the NK cell deficiency is a cellular defect in our patient.

Alternatively, the patient may have an intrinsic cellular defect of NK cells. His history of recurrent infections since infancy appears to favor this possibility. With respect to this, the patient's parents and brother had normal levels of NK cell activity (data not shown), which provided no definite evidence for familial occurrence of the NK cell deficiency.

It is noteworthy that ADCC was normal despite the absence of NK cell activity in our patient. Such a discrepancy between NK cell activity and ADCC has also been demonstrated in Hodgkin's disease.\(^18\) These findings certainly support the evidence that NK cells are distinct from K cells in ADCC,\(^2\) although they have similar cellular characteristics.\(^14\)

The clinical significance of the NK cell deficiency in our patient is of interest because NK cells provide an important defense against viral infections and tumor development.\(^2\) In fact, the patient has suffered from recurrent infections, which may be largely due to the NK cell deficiency. Further studies will determine whether such NK cell deficiency is related to the high incidence of second malignant neoplasms in Hodgkin's disease.

REFERENCES


Fig 2. NKCF activity as measured by an 18-hour \(^{3}^{1}Cr\)-release assay on K562 cells. The patient's lymphocytes, at a concentration of \(5 \times 10^6\) cells/ml, were cultured at \(37^\circ C\) for 18 hours in the absence (O-O) or presence of 1,000 U/ml recombinant IFN-\(\alpha\) (A-A) or 1,000 U/ml recombinant IL 2 (â–â–â–â–). Normal lymphocytes (â–â–â–) were cultured in an identical fashion without IFN or IL 2. After the culture, the cells were suspended at a concentration of \(3 \times 10^6\) cells/ml in culture medium containing K562 cells (\(6 \times 10^6\) cells/ml final concentration) and cultured for 4, 8, 16, and 24 hours. The cell mixtures were then centrifuged, and cell-free supernatants were obtained. The supernatants were immediately assayed for NKCF activity by an 18-hour \(^{3}^{1}Cr\)-release assay using K562 cells as a target. The patient's lymphocytes did not release detectable levels of NKCF at any time point of culture. IFN and IL 2 did not have augmentation effects on the NKCF release by the patient's lymphocytes. These experiments were performed after termination of MOPP therapy.


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