CONCISE REPORT

Human Natural Killer Cells Can Inhibit Clonogenic Growth of Fresh Leukemic Cells

By Miloslav Beran, Mona Hansson, and Rolf Kiessling

The effect of allogeneic human natural killer (NK) cells on fresh leukemic cells from three patients was investigated. The low levels of leukemic target cell lysis in the conventional $^{51}$Cr-release assay contrasted with a pronounced inhibitory effect on the colony growth of the clonogeneic leukemic target cells (L-CFC). The ability of allogeneic lymphocytes to inhibit L-CFC increased if they were pretreated with interferon (IFN), which also increased their NK activity, monitored in parallel cytotoxicity assay, against K562. Furthermore, cell separation procedures, based on differences in density among nonadherent lymphocytes, revealed that only NK cell containing fractions were inhibitory. We have also compared the susceptibility to NK-mediated L-CFC inhibition of IFN pretreated leukemic target cells with that of nontreated target cells. As in the case of NK lysis in general, this pretreatment of target cells abolished the presumably NK-mediated L-CFC inhibition. In conclusion, these data provide the first indication that NK cells can inhibit the in vitro growth of fresh clonogenic leukemia cells from patients with nonlymphocytic leukemia. The identity of NK cells as effector is strongly suggested by Percoll separation and responsiveness to interferon; the final proof awaits more sophisticated purification of these cells.

IT IS WELL ESTABLISHED that a population of human lymphocytes exerts a spontaneous cytotoxicity against a variety of human leukemic cell lines. This phenomenon is referred to as natural killer activity (NK) and is manifested by the lysis of isotope-labeled tumor target cells in a cytotoxicity assay. Experiments showing a correlation between the level of NK activity in various strains of mice and their resistance in vivo to a leukemic cell challenge suggest that NK cells indeed may play a role as a surveillance mechanism against leukemic development. Freshly derived leukemic targets are, however, usually resistant to lysis by NK cells, while lymphocytic as well as nonlymphocytic cell lines show high sensitivity. Whereas the chromium release assay measures the NK effect on the whole leukemic population, it gives no information about its acting on the clonogeneic (“stem”) cells on which the future of leukemia exclusively depends. The information on the killing by NK cells of fresh leukemic cells with clonogeneic properties would give a substantial support to the hypothetical in vivo role of NK cells against leukemia. The purpose of the present studies was (A) to study the effect of allogeneic lymphocytes on human leukemic cells capable of expressing their clonogenic potential in vitro, (B) define the effector cell, and (C) compare the results with the traditional cytolytic assay.

MATERIALS AND METHODS

The investigation was done in accordance with the Helsinki Declaration and was approved by Human Investigations Committee at Karolinska Hospital.

Patients

Leukemic cell populations used in this study were derived from previously untreated patients with acute myeloblastic leukemia (G.L.), accelerated phase of the chronic myelomonocytic leukemia (J.U.), and accelerated phase of the chronic monocytic leukemia (C.H.). All patients have had peripheral counts of leukemic cells in excess of $50 \times 10^9$ liter and grew distinct clones of leukemic cells in soft agar from both bone marrow and peripheral blood. Since the leukemic clonogeneic in vitro growth character was similar from blood- and bone-marrow-derived cells, the blood-derived ones were used for convenience.

Assay for Leukemic Clonogeneic Cells (L-CFC)

Double-layer soft agar assay with human placenta conditioned medium as the source of colony-stimulating activity (CSA) incorporated in the lower layer and assayed cells in the overlayer was used as previously described. Light density ($\pm 1.077 \text{ g/cm}^3$) mononuclear white blood cells (MWBC) containing all leukemic clonogeneic cells were used as target cells in experiments. In some experiments, MWBC were depleted of cells with surface receptors for sheep erythrocytes (E$^-$) using standard rosetting techniques and the E$^-$MWBC were used as targets. According to expected plating efficiency, $0.25-1.0 \times 10^7$ leukemic target cells were plated in 1-ml medium in 35-mm dish. Cultures were run in triplicate and scored after 8-9-day incubation at 37°C. Distinct clones of 8 and more cells were scored as L-CFC. The leukemic origin of CFC was based on in situ morphology of the L-CFC (large undifferentiated blasts in cultures from patient G.L., monocytoid blast cells from C.H., and blast and monocytoid cells in patient J.U.), absence of differentiation, and the general growth pattern. For the inhibition of L-CFC, the different effector cell (EC) populations were mixed with $10^6$ leukemic target cells (TC) in complete medium (RPMI-10% FCS), giving the desired EC/TC ratios, in a final volume of 1-2 ml. This EC:TC mixture was then incubated (37°C, 5% CO$_2$ in air) along...
with control cultures containing only target cells for 10 hr prior to the L-CFC assay.

Preparation of Effector Cells

Mononuclear white blood cells (MWBC) were obtained from heparinized whole blood, or leukocyte concentrate, from healthy volunteers, using a Ficoll-Isopaque density gradient (41.077). Phagocytic cells were removed by carbonyl iron and magnet treatment. In the experiments where fractionated EC were used, adherent cells were removed by passage of the MWBC through a nylon wool column. Nonadherent lymphocytes (NAL) were then further separated according to density on a Percoll gradient. Briefly, 15-20 x 10^7 NAL were gently layered on top of a 5-step discontinuous Percoll gradient, ranging from 42.5% (v/v) to 32.5% Percoll. The gradient was then centrifuged at 300 x g for 45 min, whereafter bands and pellet were harvested separately, washed, and counted. The cells in fractions 1-3 were considered as low density (LD) cells and were pooled to get enough cells for the experiments. This cell population contained various numbers of large granular lymphocytes (LGL), depending on experiments (40%-70%). High density (HD) cells were those harvested in the pellet; this population contained less than 1% of LGL. Total recovery was always >75% with the majority of cells in the HD fraction (~60% of input), and the LD fraction contained ~10% of the input number of cells. These different EC populations were then used both for the cytotoxicity and the L-CFC inhibition assay in parallel.

Interferon Activation of Effector Cells (EC)

Five to ten million EC were incubated for 2-4 hr at 37°C, in 1 ml medium containing 500 U of alpha-IFN (obtained from Drs. Fantes and Johnston, Wellcome Res. Lab, Beckenham). The cells were then washed 3 times and used as described for the different assays. The untreated EC were always incubated under the same conditions, but without addition of IFN to the medium. In the experiments with IFN-pretreated targets, these were incubated for 8 hr with IFN.

NK Assay

A standard 51Cr release assay in 0.2 ml Linbro microtest plates with 2 x 10^5/50 µl labeled leukemic MWBC added to 100 µl of EC and incubated for 10 hr at 37°C, 5% CO2, was used. Plates were centrifuged, 75 µl of the supernatant collected and counted in a gamma counter. The spontaneous release was determined by adding target cells to medium alone and did not exceed 20%. Total labeling was determined by resuspending the target cells before taking the 75 µl volume. Specific lysis was calculated according to the following formula:

\[
\% \text{ Specific lysis} = \frac{\text{Test counts} - \text{Spontaneous release}}{\text{Total counts} - \text{Spontaneous release}} \times 100
\]

RESULTS

Freshly isolated leukemic cells from three patients were used as targets in standard 51Cr release NK assays with allogeneic nonadherent MWBC as effector cells. Half of the effector cells was preincubated with IFN to enhance their lytic activity. No or very low levels of cytotoxicity were seen against the leukemic target cells, although the effector cells were highly active in lysing the standard NK target line K562, particularly after IFN pretreatment (Table I). In assays performed concomitantly, the same leukemic cells were tested for the number of L-CFC with or without prior exposure to the allogeneic effector cells. In all three patients, the number of L-CFC was significantly decreased after preincubation with the effector cells (Table I). Activation of effector cells with IFN further increased L-CFC inhibition, particularly in cells from patient CH in which the effect of nonactivated effector cells was moderate. We have previously reported that human NK cells in similar CFC-inhibition assays can inhibit subsequent colony formation by normal GM-CFC. We have therefore attempted to characterize the anti-L-CFC effector cells with respect to their relation to NK cells. The human NK cells have been described as large granular lymphocytes that can be enriched for among low density (LD) cells on a Percoll gradient, while the high density (HD) fraction contains the small and medium-sized NK-inactive lymphocytes. As can be seen in Fig. 1, the ability of the LD versus the HD

<table>
<thead>
<tr>
<th>Target Cells</th>
<th>Effector Cells</th>
<th>TC/EC Ratio</th>
<th>L-CFC/10^3 Target Cells (Mean ± SD)</th>
<th>Inhibition (%)</th>
<th>Target Cell Lysis† (% Specific 51Cr Release)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G.L. — AML</td>
<td>MWBC</td>
<td>—</td>
<td>860 ± 20</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>NA MWBC</td>
<td>—</td>
<td>210 ± 15</td>
<td>75</td>
<td>14.3</td>
</tr>
<tr>
<td></td>
<td>NA MWBC + IFN</td>
<td>1:5</td>
<td>105 ± 12</td>
<td>88</td>
<td>ND</td>
</tr>
<tr>
<td>C.H. — KMoL</td>
<td>E MWBC</td>
<td>—</td>
<td>990 ± 80</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>NA MWBC</td>
<td>1:5</td>
<td>774 ± 84</td>
<td>22</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>NA MWBC + IFN</td>
<td>1:5</td>
<td>114 ± 18</td>
<td>89</td>
<td>0.1</td>
</tr>
<tr>
<td>J.U. — AMML</td>
<td>E MWBC</td>
<td>—</td>
<td>540 ± 92</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>NA MWBC</td>
<td>1:4</td>
<td>328 ± 22</td>
<td>40</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>NA MWBC + IFN</td>
<td>1:4</td>
<td>215 ± 5</td>
<td>60</td>
<td>4.1</td>
</tr>
</tbody>
</table>

*Target and effector cells were coincubated for 10 hr prior to the soft agar L-CFC assay.

†The NK activity of the effector cells were assayed in 10-hr 51Cr-release assays in parallel with the L-CFC inhibition assays. Results are shown at EC/T C ratio 12:1 for K562 and at 50:1 for the patient MWBC.

ND, not determined.

Table 1. Leukemic Clonogenic Cells Are Inhibited In Vitro by Allogeneic Nonadherent MWBC, Whereas Whole Leukemic Cell Populations Is Not Lysed by Identical NK Highly Active Effector Cells
fractions to inhibit L-CFC outgrowth paralleled their NK lytic activity against the K562, with the LD fraction having a higher activity than the HD fraction (35.8% versus 5.5%). Preactivation of LD cells by IFN markedly enhanced their activity, approaching an almost complete inhibition of the clonal outgrowth, while a similar treatment of HD cells only had a marginal effect. Also, when the autologous T lymphocytes were used as effector cells, neither inhibition of the L-CFC nor any NK lysis of K562 target (2.4%) was seen. This impaired NK activity of the leukemic MWBC was apparent with all three patients (data not shown), an observation in agreement with the findings of others.9 Another characteristic of the NK system is the paradoxical effect that IFN has on the target cells, since pretreatment of target cells with IFN protects them from the lytic effect of NK cells.10,11 In line with these results we could show that pretreatment by IFN of freshly isolated leukemic cells from patient C.H. protected L-CFC from the inhibitory effect of IFN-activated effector cells. The L-CFC growth in nontreated leukemic target cells was again profoundly inhibited by IFN activated effector cells (Table 2).

**Table 2. Pretreatment of Target Cells With Interferon Protects Them From L-CFC Inhibition by Activated NK Cells**

<table>
<thead>
<tr>
<th>Treatment of Target</th>
<th>Effector Cells</th>
<th>Ratio</th>
<th>L-CFC/5 x 10^5 Targets</th>
<th>E/MWBC (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
<td>NA MWBC</td>
<td>1:5</td>
<td>387 ± 42</td>
<td></td>
</tr>
<tr>
<td>IFN</td>
<td>NA MWBC + IFN</td>
<td>1:5</td>
<td>57 ± 9</td>
<td></td>
</tr>
<tr>
<td>IFN</td>
<td>NA MWBC</td>
<td>1:5</td>
<td>297 ± 37</td>
<td></td>
</tr>
<tr>
<td>IFN</td>
<td>NA MWBC + IFN</td>
<td>1:5</td>
<td>365 ± 17</td>
<td></td>
</tr>
</tbody>
</table>

*E MWBC from patient C.H. were used as target cells after 8-hr incubation with or without 1000 U IFN.

† Allogeneic nonadherent MWBC with a lytic NK activity against K562 of 17.4% without and 62.6% after IFN activation were used as effector cells.

**DISCUSSION**

NK cells are mainly considered as a first line of defense against malignant cells, but recent evidence has also indicated that they may be involved in the regulation of hematopoietic functions.12,13 Thus, we have reported that NK cells can lyse certain primitive cells contained within the fetal hematopoietic and thymic tissue of mice and man.14,15 Recently, we have also demonstrated that both autologous and allogeneic NK-enriched but not NK-depleted nonadherent lymphocytes interfere with the ability of granulocyte-macrophage committed stem cells (GM-CFC) to form...
clones in semisolid media. The inhibition of GM-CFC is notably enhanced by IFN and appears to be at least partly mediated by cell–cell contact.

The new finding in the present report shows that a similar mechanism may be inhibiting the clonal outgrowth of L-CFC. The active cell type showed all the characteristics of an NK-cell: it is present in normal healthy individuals among the nonadherent MWBC; it is strongly enriched among low density cells; its inhibitory capacity is markedly enhanced when preactivated with IFN; and IFN has the paradoxical effect of protecting the L-CFC. Yet, in confirmation of results from others, the whole leukemic cell population proved markedly resistant to direct lysis by highly NK-enriched and IFN-activated cell populations. We are considering two possible explanations to this seemingly contradictory finding. First, since the leukemic population is heterogenous, NK cells may lyse only a minor population that is undetectable by our isotope release assay. This true target population could correspond to the more primitive clonogenic population detected by the L-CFC assay. Alternatively, L-CFC are inhibited by an NK-mediated mechanism different from the lytic action of NK cells. This mechanism may be related to the fact that NK cells are endowed with the ability to produce interferons when triggered by contact with tumor cells. Thus, IFN, or other soluble factors, synthesized by NK cells during the L-CFC assay may exert a cytostatic effect on the L-CFC. The interferon production could be triggered by the preincubation and direct cell–cell contact with leukemic cells. Taken together, the presented data strongly favors the NK cell population as the one responsible for the observed antileukemic activity. The final proof, however, can only be made when completely pure NK population(s) become available.

NK cells have been shown to be activated in human BM graft recipients with acute GVHD. This finding could pertain to the present observation of an anti-L-CFC effect by NK cells, since in leukemic patients, the relative relapse rate was described to be 2.5 times less in allogeneic marrow recipients with GVHD as compared to those without it. The finding that both native and IFN-activated NK cells inhibit preferentially leukemic clonogenic cells gives further support for the role of NK cells in surveillance of leukemia, as inferred from extensive data in mouse experimental models.

REFERENCES

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