Stimulation of Natural Killer Cell Activity and Inhibition of Proliferation of Various Leukemic Cells by Purified Human Leukocyte Interferon Subtypes

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ABSTRACT

One of several human leukocyte interferon subtypes A (LeIF-A), obtained in purified form from a gene cloned in Escherichia coli, stimulated human peripheral blood natural killer cell activity, whereas another human leukocyte interferon subtype D (LeIF-D) had no effect with the use of K562 as target cells. With Daudi as target cells, both LeIF-A and LeIF-D stimulated natural killer cell activity. A hybrid human leukocyte interferon, NH2-terminal 61 amino acids and COOH-terminal 104 residues of LeIF-A and LeIF-D, respectively (LeIF-AD) showed greater stimulation than did LeIF-A, but the stimulation did not exceed that of natural buffy coat interferon. A mixture of equal antiviral units of LeIF-A and LeIF-D was no more effective than was LeIF-A alone. The cloned interferon subtypes showed differential effects on the proliferation of three human leukemic cell lines: Daudi (B-cell lymphoblastoid leukemia); BALL 1 (B-cell acute lymphoblastic leukemia); CCRF-HSB-2 (T-cell acute lymphoblastic leukemia). Growth of Daudi cells was generally most sensitive to all the interferons tested, LeIF-A, -D, -AD, and auffy coat preparation; no viable cells remained after 120-hr exposure to 1000-unit/ml doses of the interferons. BALL 1 was relatively resistant to the interferon subtypes tested including LeIF-AD, but this cell line was very sensitive to a preparation of natural buffy coat interferon. CCRF-HSB-2 showed some sensitivity to all the interferons with greatest sensitivity to LeIF-A (10% of the viable cells were detected after 1000 units/ml exposure for 120 hr). In contrast to the leukemic cell lines tested, human amnion cells (WISH) and the human erythroid leukemia, K562, were resistant to the antiproliferative activity of the interferons.

INTRODUCTION

Although interferons were identified by their antiviral effects, antiproliferative effects have also been demonstrated (8). Impure interferon preparations have shown pronounced effects against tumor cells in culture and experimental tumors in animals (8, 19). Direct cytostatic and cytotoxic effects may occur against tumor cells, but indirect effects mediated by parameters of the immune system are important (7); of these, stimulation of NK1 cell cytotoxicity has been demonstrated in vitro and in vivo (4, 13, 14). The relationship between antitumor and antiviral effects of interferons is not clear, and it is possible that the relative contribution of the various mechanisms is different for different tumors.

Cloning of human leukocyte interferon genes in bacteria by recombinant DNA methods has already revealed the existence of 8 distinct leukocyte interferon subtypes, and annealing of DNA probes to the human genome indicates the presence of at least 10 genes (6). These leukocyte interferon subtypes have 165 or 166 amino acid residues and at least 73% homology in their amino acid positions (6). The frequency of cloning these genes from complementary DNA prepared from mRNA is likely to approximate the frequency of the interferon subtypes in natural preparations. On this basis, 2 subtypes, LeIF-A and LeIF-D, may constitute almost 70% of the various subtypes in natural preparations (6).

The various human leukocyte interferon subtypes revealed by recombinant DNA methods have been found to have distinct antiviral properties in cell cultures (21). In addition, new hybrid interferons have been obtained by splicing 2 genes at internal restriction enzyme sites, and these also have distinct antiviral properties (22). One such hybrid interferon, LeIF-AD (comprising the 61 NH2-terminal residues of LeIF-A and the 104 COOH-terminal residues of LeIF-D), has been obtained in highly purified form (98%). This interferon and similarly purified preparations of LeIF-A and LeIF-D are here compared in terms of antitumor activity against various human tumor cell lines and ability to stimulate NK cell activity in human PBL preparations.

MATERIALS AND METHODS

Lymphocytes. Human PBLs of normal volunteers were prepared from leukopheresis preparations by Ficoll (Pharmacia, Piscataway, N. J.) gradient centrifugation as described elsewhere (2). The PBL preparations contained more than 95% medium-sized mononuclear cells.

Interferons. The amino acid sequences of LeIF-A and -D have been reported elsewhere (6, 23). LeIF-A was purified as outlined elsewhere (23), and a similar procedure with the addition of the monoclonal antibody affinity step was used for LeIF-D (16). The hybrid interferon, LeIF-AD, was similarly purified (purification procedure will be reported elsewhere). Interferon titers are given in International Reference Units (NIH Human Leukocyte Interferon Reference Standard GO 23-901-527). The bacterial-derived interferon preparations were greater than 95% pure and electrophoretically homogeneous and had specific activities of 2.7 × 109 (LeIF-A), 3.2 × 109 (LeIF-D), and 1.9 × 109 (LeIF-AD) units/mg protein when titrated in human amnion cells (WISH) with vesicular stomatitis virus as challenge. A preparation of natural human buffy coat interferon was obtained from the Wadley Institute, Dallas, Texas, and this had a specific activity of 1 × 109 IU/mg.

Cells. K562 (human erythroid leukemia), Daudi, and BALL 1 cells (B-cell lymphoblastoid and acute lymphoblastic leukemias, respectively) were obtained from Dr. J. Minowada of Roswell Park Memorial Institute, Buffalo, N. Y., and were grown in Roswell Park Memorial Institute medium (Grand Island Biological Co., Grand Island, N. Y.).
containing 10% fetal bovine serum at 37°. CCRF-HSB-2 (T-cell acute lymphoblastic leukemia) was obtained from American Type Culture Collection, Rockville, Md., and maintained in minimum essential medium (Grand Island Biological Co.) with 10% fetal bovine serum. WISH cells (human amnion) were obtained from Dr. P. Trown, Hoffmann-La Roche Inc., Nutley, N. J., and maintained in minimum essential medium with 10% fetal bovine serum.

**NK Cell Activity.** Target cells (K562 or Daudi) were labeled with 51Cr by incubating 6 x 10^6 cells in 0.2 ml medium with 200 μCi of sodium [51Cr]chromate (specific activity, 5 to 40 mCi/mg chromium; Amersham Corp., Arlington Heights, Ill.) for 45 min at 37°. Such labeled target cells (1 x 10^6) were incubated for 4 hr at 37° with varying numbers of interferon-treated lymphocytes. Lymphocytes were treated with interferons for 1 hr at 37° prior to incubation with the target cells. After incubation, the samples were centrifuged at 12,000 x g for 10 sec, and the radioactivity in 0.1 ml of supernatant was measured in a γ-scintillation spectrometer. Each assay was replicated 6 times. Spontaneous 51Cr release, which was the measurement of the radioactivity after incubation in the absence of lymphocytes, was approximately 10% of the total 51Cr incorporated into the target cells. The percentage of cytolyis was calculated as follows:

\[
\text{% of cytolyis} = \frac{\text{Test cpm} - \text{spontaneous cpm}}{\text{Total cpm} - \text{spontaneous cpm}} \times 100
\]

**Cytotoxicity Assays in Culture.** Exponentially growing cell preparations were incubated at 37° in an atmosphere containing 5% CO2 in the presence or absence of interferon. Cell growth was determined at various times by either hemocytometer counts of viable cells using trypan blue dye exclusion (Daudi, BALL 1, K562 and CCRF-HSB-2) or measuring the absorbance after staining monolayers with crystal violet using a Microelisa Autoreader, MR580 (WISH cells). WISH cells were grown in microtiter plates (Costar, Cambridge, Mass.), and cell numbers were estimated from a calibration curve relating cell numbers to absorbance at 570 nm. Hemacytometer counts were carried out in triplicate, and 8 estimates of the number of WISH cells were made from the absorbance data.

**RESULTS**

The mean cytolytic activity of PBLs from 5 individuals against K562 target cells is shown in Table 1A for various interferon preparations. The increase in cytolytic activity at interferon concentrations above 10 IU/ml is relatively small in the purified interferon species. With the buffy coat interferon preparation, a progressive increase in lysis occurred up to 1000 IU/ml. With all the interferon preparations tested, the highest dose (10^4 IU/ml) seemed to cause slightly less lysis than did 10^3 IU/ml. Only the data for a target:effector cell ratio of 100 are shown, and the results with ratios of 1:50 and 1:25 were similar but less in magnitude. LeIF-D was ineffective as a stimulator in the assay conditions used against K562. The PBL-stimulatory activity of LeIF-AD was higher than that of LeIF-A or mixtures of LeIF-A and LeIF-D. Analysis of variance of the data (Table 1A) for interferon, 10^3 IU/ml, showed significant differences at p < 0.05, with LeIF-AD significantly more effective than LeIF-A, -D, or the mixture of LeIF-A and -D. The mixture of LeIF-A and LeIF-D (equal number of units) was generally less active than LeIF-A and the hybrid interferon, LeIF-AD. Using Daudi cells as targets, LeIF-D showed marked stimulation of NK cell activity, as found by Masucci et al. (9), and LeIF-A and -AD were also active in the order: LeIF-AD > -A > -D (see Table 1B).

To determine the relative sensitivities of a normal and a leukemic cell line to cytotoxic effects of the interferon preparations, the growth of Daudi and WISH cells were compared in the presence of varying concentrations of the interferons. As shown in Table 2, inhibition of Daudi cells by a 10-unit/ml dose of an interferon was approximately 50% after 72 hr. Significantly greater inhibition of cell growth did not occur with higher concentrations of the interferons. In contrast to the leukemic cell (Daudi), human amnion cells (WISH) were essentially resistant to all the interferons tested; no significant growth inhibition by an interferon less than 1000 units/ml was observed.

### Table 1

**Percentage of activation of NK cell activity in PBLs by various interferon preparations**

See "Materials and Methods" for experimental detail. Target:effector cell ratio, 1:100.

<table>
<thead>
<tr>
<th>Interferon (IU/ml)</th>
<th>LeIF-A</th>
<th>LeIF-AD</th>
<th>LeIF-D</th>
<th>LeIF-A + D</th>
</tr>
</thead>
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<tr>
<td><strong>A. K562</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>14.8 ± 2.7^a</td>
<td>14.8 ± 2.7</td>
<td>14.8 ± 2.7</td>
<td>14.8 ± 2.7</td>
</tr>
<tr>
<td>10^1</td>
<td>19.3 ± 3.0</td>
<td>17.0 ± 3.6</td>
<td>14.0 ± 1.6</td>
<td>11.0 ± 1.2</td>
</tr>
<tr>
<td>10^2</td>
<td>22.2 ± 3.8</td>
<td>21.6 ± 4.1</td>
<td>18.6 ± 2.0</td>
<td>14.0 ± 1.8</td>
</tr>
<tr>
<td>10^3</td>
<td>24.4 ± 3.6</td>
<td>22.8 ± 1.9</td>
<td>20.4 ± 2.2</td>
<td>16.8 ± 2.1</td>
</tr>
<tr>
<td>10^4</td>
<td>18.0 ± 1.9</td>
<td>20.5 ± 1.0</td>
<td>14.5 ± 1.6</td>
<td>13.0 ± 0.6</td>
</tr>
<tr>
<td><strong>B. Daudi</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>14.8 ± 2.7^a</td>
<td>14.8 ± 2.7</td>
<td>14.8 ± 2.7</td>
<td>14.8 ± 2.7</td>
</tr>
<tr>
<td>10^1</td>
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<td>20.5 ± 1.0</td>
<td>14.5 ± 1.6</td>
<td>13.0 ± 0.6</td>
</tr>
</tbody>
</table>

^a Mean ± S.E.
Because the various cloned interferon subtypes differ in their antiproliferative effects, we compared their activities with that of a buffy coat interferon preparation at the highest concentration (10,000 IU/ml). The results (Table 2) show that, at this concentration, buffy coat interferon is as potent as is LeIF-A against Daudi cells but more potent than are LeIF-A and LeIF-D against WISH.

The relative sensitivities of the human leukemic cells (Daudi, BALL 1, CCRF-HSB-2, and K562) to the growth-inhibitory effect of the interferon preparations were compared at interferon concentrations of 1000 IU/ml. As shown in Chart 1a, Daudi cell growth was the most sensitive of the 4 cell lines to the 3 recombinant DNA-derived interferons, LeIF-A, -D, and -AD, and buffy coat interferon appeared to have comparable activity. Further cultivation of these cells, with or without interferon, showed that all treated cells were nonviable after 120 hr in the presence of the interferon preparations. In contrast to Daudi, BALL 1 cells were relatively resistant to LeIF-A, -D, and -AD (Chart 1b) and were persistently growing at 120 hr of exposure with only slightly reduced growth rates. However, proliferation of BALL 1 cells was greatly inhibited by buffy coat interferon. The number of BALL 1 cells after a 72-hr exposure to buffy coat interferon was less than that originally seeded. LeIF-A, -D, -AD, and buffy coat interferon showed considerable variation in activity on the growth of CCRF-HSB-2 (Chart 1c). The relative inhibitory activities of all the interferon preparations tested in this cell line were LeIF-A > buffy coat interferon > LeIF-A + LeIF-D > LeIF-AD > LeIF-D with approximately 50% inhibition of the cell growth by LeIF-D. Among the leukemic cells tested, K562 cells were most resistant to antiproliferative activity of the interferons, and growth of the cells was not significantly affected by any of the interferons (Chart 1d).

The inhibitory effect on cell growth could be observed only after continuous exposure for at least 6 hr, regardless of the interferon used (data not shown). Because of this delayed effect, it seemed possible that growth inhibition occurred indirectly via some factors produced by the treated cells themselves. To test this possibility, we treated Daudi cells with up to 10,000 IU of LeIF-AD per ml for 48 hr, and after the cells were washed, they were resuspended in fresh medium. The medium from these washed, resuspended cells was removed after 52 and 78 hr and tested for its ability to inhibit fresh cultures of
Daudi cells. The supernatants showed no inhibition of growth of Daudi cells over 120 hr (data not shown).

**DISCUSSION**

The present studies demonstrate that highly purified human leukocyte interferon subtypes produced in bacteria enhance natural killing activity of human PBLs and suppress the growth of neoplastic cells in culture. Where these effects are observed, they occur at relatively low interferon concentrations (10 IU/ml) with only slight increases at higher concentrations. Of the tumor cell lines tested, Daudi seems the most sensitive to the antiproliferative effects of the purified interferon subtypes, and the magnitude of the effect is essentially the same for the various subtypes and for the preparation of naturaluffy coat interferon. In contrast, the purified interferon subtypes have virtually no effect against another B-cell tumor line, BALL 1, although the buffy coat interferon preparation is very potent against this tumor cell line. Some variation is observed in the potency of the various interferon preparations against the T-cell tumor cell line CCRF-HSB-2, and in this case the LeIF-A subtype is at least as effective as the buffy coat interferon preparation.

The absence of significant antiproliferative effects with the purified interferon subtypes against BALL 1, despite a pronounced effect with the buffy coat interferon preparation, may be accounted for in several ways. One or more of the other leukocyte interferon subtypes may be the active component that is present in the mixture which constitutes the natural interferon preparation. Possibly one of the other subtypes, in combination with LeIF-A or LeIF-D, is necessary for antiproliferative effects against BALL 1. Although interactive effects between the interferon subtypes have been observed in antiviral assays (18), mixtures of LeIF-A and LeIF-D are no more effective than either subtype in the present studies. Alternatively, the pronounced antiproliferative effect of the buffy coat interferon against BALL 1 is primarily due to contaminating materials in this preparation. However, this would not seem to be the case generally because equal or greater antiproliferative effects are observed with the subtypes, compared with the buffy coat preparation, against the Daudi and CCRF-HSB-2 cell lines.

Indirect effects are known to be of importance even for antiviral effects of interferons in vivo (12, 17). This is true also for a leukocyte interferon subtype. LeIF-A has relatively low antiviral activity in rabbit cell cultures but is as potent as buffy coat preparations in Herpesvirus type 1 infections of the rabbit eye. Whether the same indirect mechanisms are involved in antiviral and antiproliferative effects is uncertain. Comparison of these effects is difficult, but the brief exposure necessary to confer antiviral activity contrasts with the prolonged periods necessary to observe antiproliferative effects. The possibility that delayed antiproliferative effects are mediated by factors released by interferon treatment seems to be excluded by the present studies. The same indirect mechanisms could be involved in antiviral and antitumor effects, although the differential antiproliferative effects against normal and neoplastic cells requires consideration. The apparent protective effect of interferons against NK cell lysis of fibroblasts may enhance the

**REFERENCES**


**ACKNOWLEDGMENTS**

We thank Drs. D. Estell, E. Rinderknecht, and C. McGregor for the supply of purified interferons and A. Hui for help in lymphocyte preparation.
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1316  CANCER RESEARCH VOL. 42
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