Effects of Recombinant and Hybrid Recombinant Human Leukocyte Interferons on Cytotoxic Activity of Natural Killer Cells*

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Two recombinant human leukocyte interferons (A and D), five hybrid interferons containing varying portions of A and D, and one fibroblast recombinant interferon were tested over a wide range of concentrations for their ability to modulate cytolytic activity of human natural killer (NK) cells. All of the interferons tested were purified to homogeneity. Although all the interferons were active, there were significant quantitative differences in their ability to augment cytolyis and the rank order of potency was reproducible among donors. The various recombinant interferons were also tested for their ability to augment mouse NK activity and the parental D and the A/D hybrids exhibited significant augmentation of cytolyis, which was consistent with their interspecies reactivity in viral neutralization assays. There generally was a direct correlation between antiviral activity and the ability of interferon to augment mouse NK activity; however, this correlation was not evident when tested on human cells. The study of these hybrids led to the identification of two molecules (A/D Bgl and A/D Pvu) which are very active in augmenting mouse NK activity. In addition, considerable insight has been obtained regarding the structure-function relationship of these leukocyte interferons and their ability to boost murine NK. This biological activity was associated with the COOH-terminal portion of the D interferon.

Human leukocyte interferons consist of many individual species (1, 2), with differing amino acid sequences (3-5). In addition, several natural and recombinant molecules have been purified and characterized (6-8). Because of major homologies among the individual leukocyte interferons, it has been possible to construct hybrids from IFN-αA1 and IFN-αD (8). These hybrid recombinant interferons exhibit characteristic antiviral activities on human, bovine, mouse, feline, and rat cells and they also vary in other biological effects (8-10).

The antitumor effects of interferon are mediated by either or both of two mechanisms: 1) direct cytotoxic effects on tumor cells and 2) modulation of host defense mechanisms (11-17). Among the various effects of interferons, their ability to rapidly augment cell-mediated cytotoxicity by NK cells and monocytes (18-26) has been studied in great detail. We have recently demonstrated that homogeneous species of human leukocyte interferons could appreciably augment the reactivity of NK cells (25). In addition, we have also demonstrated that the various homogeneous natural leukocyte interferons (27) vary considerably in their potency in augmenting human NK cells (28). Since the native homogeneous molecules of IFN-αA and IFN-αD differ appreciably in their ability to augment NK cytotoxicity and in their interspecies cross-reactivity with mouse cells, the evaluation of the effect of these genetically constructed hybrid recombinant leukocyte interferons on cytotoxicity by NK cells offers several interesting opportunities. 1) The ability to determine the structure-function relationships regarding the possible association between the presence of various portions of IFN-αA and IFN-αD in the hybrid molecules and the expression of various biological functions. 2) The development of interferon molecules with increased biological potency. 3) The identification of human interferon(s) with potent activity in mice, providing an excellent model system to investigate in depth the therapeutic and other in vivo effects of human interferon. 4) The potential identification of an interferon with highly selective biological effects (i.e. the ability to boost defined populations of effectors), which might aid in the determination of the relative roles of interferons in in vivo therapeutics.

EXPERIMENTAL PROCEDURES

Materials

Interferon Preparations—Species of human recombinant leukocyte IFN-αA, IFN-αD, and hybrids of A and D produced by the use of Bgl and Pvu restriction enzymes (IFN-αA/D Bgl, IFN-αA/D Pvu), IFN-αD/A (Bgl), IFN-αD/A (Pvu), IFN-αA/D/A and recombinant fibroblast interferon were prepared as previously described (4-6, 8, 27). All preparations were homogeneous, with amino acid sequences as reported elsewhere (5, 8). In addition, a virus-induced mouse (α/β) interferon provided by the laboratory of the late Dr. K. Paucker was employed. Interferon titers were determined using an assay of inhibition effects (25, 27) on bovine MDBK and human AG-1732 cells.

Effector Cells—Peripheral blood mononuclear cells were prepared from heparinized blood of normal, adult human donors by centrifugation on a Ficoll-Hypaque density gradient (28). Effector cells from donors previously found to show consistent boosting of activity by partially purified interferon were used for these studies. To obtain a subpopulation of cells that were enriched for NK activity, human peripheral blood mononuclear leukocytes were further separated by centrifugation on a discontinuous gradient of Percoll (Pharmacia Chemicals, Uppsala, Sweden) as previously described (30). The fractions containing a high percentage of large granular lymphocytes, the small subpopulation of cells shown to account for all NK and ADCC activities in peripheral blood (30), were then tested. C3H mouse spleen cells, depleted of macrophages by passage over nylon columns, were used as the source of mouse NK cells. (No Percoll separation was employed.)

Target Cells—K-562, a cell line derived from a patient with chronic
myelogenous leukemia in blast crisis (31) and YAC-1, a cell line derived from a mouse T cell lymphoma (32), were used as the target cells for human and mouse NK activity, respectively.

Methods

Treatment with Interferon—To assess the effects of interferon on NK activity, various concentrations of interferon, in RPMI 1640 medium containing 10% fetal bovine serum, were incubated in a volume of 1 ml with effector cells (1-2.5 x 10^6/ml) for 2 h at 37°C, which were then washed before testing.

Assay for NK Activity—As previously described (32), tests were performed at several effector:target cell ratios and the data were expressed as lytic units as defined by linear regression of the dose-dependent portion of the curve.

Considerable variation in boosting among donors with these and other interferons. All samples were tested simultaneously and the relative rank order remained constant; however, the augmented dose often varied considerably (see Table I).

The AD50 did not give a different pattern of results from those found by use of the 50% maximal dose, since the dose-response curves in individual experiments were parallel. We chose the AD50 calculation to provide an indication of the minimal dose required to induce a significant degree of boosting. These doses may be more useful for application of these results to estimation of the amounts of interferon needed for clinical trials.

RESULTS

The homogeneous species of recombinant leukocyte and fibroblast interferons were tested for their ability to modulate NK activity. All of the interferons were tested multiple times with the selected normal donors in the NK assay. Fig. 1 illustrates typical results with several of the recombinant species and demonstrates the augmentation of NK activity by IFN-αD and 2 of the hybrid species. The IFN-αA/D (Bgl) recombinant significantly augmented the cytolytic activity above the control, demonstrating an AD50 from regression analysis of dose curve in this experiment of 3.5 units of interferon, whereas the IFN-αD demonstrated a significant augmentation at 10 units of interferon. The hybrid IFN-αA/D/A was a relatively weak booster of NK cytotoxicity (with an AD50 of 200 units of interferon).

The various hybrid molecules demonstrated large quantitative differences in their ability to augment the reactivity against human NK cells (Table I). IFN-αA/D (Bgl) consistently gave high levels of augmentation (with a mean AD50 of 1.3 units of interferon). In addition, the IFN-αD/A (Puu) and the IFN-αD/A/B gl hybrids were quite efficient boosters compared to less augmenting effects of the IFN-αDA/D (Puu) and the IFN-αA/D/A molecules. Quite interestingly, recombinant human fibroblast interferon was consistently a high level booster for human NK activity, with potency quite similar to the IFN-αA and IFN-αA/D (Bgl) leukocyte interferons.

Similar experiments were performed to determine the possible effects of the various recombinant and hybrid recombinant interferons on human NK activity with interferons.

![Fig. 1. Augmentation of human NK activity with interferons. The basal activity as well as the 50% increase level (hatched area) are indicated and results are shown with several interferons: IFN-αA/D/Bgl, △ IFN-αA/D, ○ and IFN-αA/D/A. □ Cytotoxic activity was measured against K562 in a 4-h Cr release assay and expressed in lytic units at 30%.](image)

![Fig. 2. Augmentation of mouse NK activity with interferons. The basal activity as well as the 50% increase level (hatched area) are indicated and results are shown with several interferons: mouse α interferon, △ IFN-αA/D Bgl, ○ and IFN-αA/D/A. □ Cytotoxic activity was measured against YAC-1 target cells, in a 4-h Cr release assay and expressed in lytic units at 15%.](image)
nant molecules on mouse NK activity and Fig. 2 illustrates some of these results. For comparison, a preparation of mouse interferon also was tested (which demonstrated on ADso of 750 units of interferon). The IFN-αA/D/A hybrid, which has no appreciable antiviral activity on mouse cells (8), demonstrated no augmentation of mouse NK activity even at 10,000 units of interferon. In contrast, the A/D (Bgl) hybrid, which has been reported to have antiviral (8) activity on mouse cells, demonstrated fairly strong augmentation of mouse activity (an ADso of 100 units of interferon).

Table I provides a summary of the effects of IFN-αA, IFN-αD, and the hybrid interferons on mouse NK activity. Mouse α interferon was used as a positive control in all experiments with mouse NK cells. Only the recombinant D interferon and the A/D hybrids demonstrated activity on mouse NK cells, whereas the other molecules consistently caused no augmentation of mouse NK activity, even at 10,000 units of interferon.

Because of the ability to perform such experiments with homogeneous materials of known molecular weight, one can calculate the number of molecules per cell required to obtain augmentation of other biological effects (5, 8, 35). Although it has been conventional to present data with interferon in terms of units of interferon activity relative to a reference standard, we have attempted here to relate functional activity with molecular activities of interferon, as molecules per cell needed to induce 50% augmentation of natural cytotoxicity. Such expression of the data in these absolute terms should provide the basis for a clearer evaluation of activity profiles than can be conventionally obtained with the use of antiviral interferon units, with impure interferons, since it is conceivable that the impurities may appreciably modulate functional activity. We would expect that analysis of the data in terms of molecules required per cell will more reliably compare the effects of each of the interferons on the activities of various types of effector cells.

Table II summarizes the results of these calculations in regard to boosting of human and mouse NK activity. In regard to the number of molecules of interferons necessary for an ADso for human NK activity, considerable heterogeneity was seen among the interferons. IFN-αA was very potent with an ADso of 120 molecules. In contrast, the other parental molecule, IFN-αD, required approximately 300,000 molecules/cell for stimulating NK activity. Of interest was that all of the hybrid molecules were at least as effective as the IFN-αD parental molecule, but only the IFN-αA/D (Bgl) had potency similar to that of IFN-αA. If one ranks the potencies of the various interferons on a molecular basis, the following order was seen with boosting of human NK activity: (A/D (Bgl) ≈ A > A/D (Puu) > A/D/A ≈ D/A (Puu) > D/A (Bgl) ≈ D).

The ranking of the human interferons with respect to their ability to boost NK activity in terms of antiviral units on human cells (8) was: A ≡ A/D (Bgl) > D ≡ D/A (Puu) > D/A (Bgl) > A/D (Puu) ≡ A/D/A. Relatively speaking, the general order seems to be similar, with the IFN-αA and the IFN-αA/D (Bgl) being quite potent, whereas the IFN-αD and the D/A hybrids were less active in each assay. These differences became most evident when the calculations were made in terms of numbers of molecules required for similar effects.

With regard to effects on mouse NK activity, the A/D hybrids were found to be substantially more potent than either of their recombinant parental molecules, with the D molecule showing a low level of cross-reactivity, requiring a large amount of interferon. The efficiency of the IFN-αA/D (Bgl) and the IFN-αA/D (Puu) species in inducing augmentation of mouse NK activity was similar to their potent antiviral effects on mouse cells (8).

Because of the ability to calculate the molecules of interferon needed, the antiviral to NK boosting ratio can be calculated. Based on the molecules needed to induce antiviral effects (8) and the data present in this report, considerable variations can be seen (Table III). The A/D (Bgl) is unique in the ability to dissociate the AV and NK abilities with a 95-fold ratio. The IFN-αA parental molecule also shows an increased ratio of 41. This associates an increased NK augmenting capacity with the IFN-αA and the IFN-αA/D (Bgl) hybrid and separates their activities as unique from the other molecules.

**DISCUSSION**

The structures and restriction maps of the coding regions of the native proteins and the recombinant hybrids, joined at the BglII and PvuII restriction enzyme sites, have been diagrammatically reported (8). The cross-reactivity of IFN-αD with mouse cells, along with several hybrid recombinants that

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**TABLE I**

**Number of molecules of the various recombinant interferons required for boosting of NK activity**

<table>
<thead>
<tr>
<th>Species of IFN-α</th>
<th>Concentration of IFN at ADso</th>
<th>Molarity at ADso</th>
<th>Required number of molecules/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pg/ml</td>
<td>pM</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>9.4</td>
<td>9.48</td>
<td>120</td>
</tr>
<tr>
<td>D</td>
<td>2.3 × 10^6</td>
<td>1.172</td>
<td>2.8 × 10^6</td>
</tr>
<tr>
<td>A/D (Bgl)</td>
<td>4.6</td>
<td>0.24</td>
<td>58</td>
</tr>
<tr>
<td>A/D (Puu)</td>
<td>105.2</td>
<td>5.41</td>
<td>130</td>
</tr>
<tr>
<td>D/A (Bgl)</td>
<td>2.0 × 10^6</td>
<td>1.041</td>
<td>2.5 × 10^6</td>
</tr>
<tr>
<td>D/A (Puu)</td>
<td>3703</td>
<td>193</td>
<td>4.6 × 10^4</td>
</tr>
<tr>
<td>A/D/A</td>
<td>1628</td>
<td>84.7</td>
<td>2.0 × 10^5</td>
</tr>
</tbody>
</table>

**TABLE II**

**Specific molecular activities for antiviral and NK cell activities**

The molecules/cell for NK cell activity are taken from the data of Tables I and II and that for 50% inhibition of antiviral activity (AV) from Rabberg et al. (8). The ratio of the molecules/cell for antiviral activity to that for natural killer cell activity (AV/NK) was calculated from the mean values. All antiviral units for the human interferons are expressed as the antiviral activity on AG1732 human fibroblasts.

**Table**

<table>
<thead>
<tr>
<th>Interferon</th>
<th>Mean</th>
<th>Range</th>
<th>NK</th>
<th>Antiviral</th>
<th>AV/NK</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>120</td>
<td>(40-400)</td>
<td>4900</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>2.8 × 10^6</td>
<td>(2.1 × 10^6-5.6 × 10^6)</td>
<td>3.6 × 10^4</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>A/D (Bgl)</td>
<td>58</td>
<td>(45-223)</td>
<td>5500</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>A/D (Puu)</td>
<td>1300</td>
<td>(325-3250)</td>
<td>4100</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>D/A (Bgl)</td>
<td>2.5 × 10^6</td>
<td>(2500-6.3 × 10^6)</td>
<td>1.5 × 10^5</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>D/A (Puu)</td>
<td>4.6 × 10^6</td>
<td>(1.4 × 10^6-3.2 × 10^6)</td>
<td>5.9 × 10^4</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>A/D/A</td>
<td>2.0 × 10^6</td>
<td>(1.1 × 10^5-5.7 × 10^5)</td>
<td>3.6 × 10^4</td>
<td>1.8</td>
<td></td>
</tr>
</tbody>
</table>

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exhibited antiviral activity on mouse cells (8, 33), has been extended here to their augmenting effects on mouse NK activity. An examination of the data in relation to the structures of the hybrids provides some insight into the portion of the interferon molecule required for crossing the species barrier. Comparison of the IFN-αA/D and the IFN-αD hybrids indicates that the distal (COOH-terminal) portion of the D molecule is essential for biological activity on mouse NK cells. This conclusion also applied to the antiviral activities of these interferons (8). Furthermore, comparison of the data with the IFN-αA/D (Bgl) and IFN-αA/D (Pvu) hybrids, which differ only by three amino acid substitutions at positions 69, 80, and 86, indicates that the IFN-αD form of this region confers a 2-fold increase in potency on a molar basis.

With regard to their effects on human NK cells, the species of human leukocyte interferons also varied quite substantially in the relative number of molecules required to induce similar levels of augmentation of cytotoxic activity. The relatively low activity of IFN-αD and both D/A hybrids in stimulating NK activity of human cells corresponds to their low antiviral activity on human cells. Apparently, introduction of amino acids corresponding to IFN-αD in the NH2-terminal portion of the molecule reduces its activity on human cells. If one assumes that the biological activity of interferon is dependent on its ability to interact with a specific cell surface receptor, one could attribute the differences in activity to the conformationally altered binding affinity of the various hybrids to the cellular receptors for interferon.

Comparison of the antiviral and antiproliferative activities of these hybrid molecules with their NK activities indicated a similar heterogeneity of effects on mouse and human cells (8). The antiviral activities on human cells of IFN-αA/D (Pvu), IFN-αA/D (Bgl), and IFN-α were similar, with the IFN-αD and the D/A hybrids having lower potency. Regarding antiproliferative activity on the human Daudi B cell line, the results were quite similar, with the order of decreasing potency being IFN-αA/D (Bgl), IFN-αA, and IFN-αA/D (Pvu). It is of interest that the relative potencies of the various hybrids differed substantially among the assays. Although IFN-αA/D (Bgl) and IFN-α were highly immunomodulatory on human NK cells, the IFN-αA/D (Pvu) hybrid was considerably less potent in its ability to augment cytolytic activity (Tables I and II). As a further contrast among the assays, both D/A hybrids, which were reported (8) to have a low potency in the antiviral and antiproliferative assays were fairly effective in the NK system (Tables I and II). Regarding mouse activities, the relative potencies of the hybrids in the different systems seemed very similar, with IFN-αA/D (Bgl), IFN-αA/D (Pvu), and IFN-αD ranking 1, 2, 3, respectively, in both assays. The remainder of the hybrids could not be ranked in the cytolytic assay because of their inactivity at the highest concentration tested.

The data of Table III compare the specific molecular activities of the parental and hybrid interferons for stimulation of NK cells and for the antiviral effect. It is evident that IFN-αA, IFN-αA/Bgl, and IFN-αD/Pvu require much less interferon to stimulate natural killer cells than to protect cells from virus infection, whereas IFN-αD, IFN-αA/Pvu, and IFN-αD/A exhibited similar specific molecular activities for both effects. This large difference in the AV/NK ratios (Table III) for A/D Bgl of nearly 100-fold supports the suggestion that the antiviral activity and ability to stimulate NK cells are mediated by different mechanisms. These results indicate that the biological effects of the interferons can be dissociated and are due to different molecular mechanisms. The existence of multiple interferon receptors that respond differentially to each of the interferons could account for the differences in the molecular antiviral/natural killer cell activity ratios. Alternatively, it is possible that the interferon receptor is a complex one that can differentially modulate various molecular pathways.

The ability to clone and express the genes for the various species of interferon has made it feasible to produce these interferons in large quantities. It is possible that more finely tuned engineering of the interferon molecules might lead to a clear identification of the portions of the sequence responsible for each activity and to the development of molecules exhibiting only one of the various activities that are associated with interferon. The large scale production of such molecules would allow studies to determine the relative importance of each of the biological activities for the proposed in vivo effects of interferon. For example, one can envision cancer therapy with a biologically active interferon that has been engineered to minimize associated toxicities. Finally, it is important to note the potential value of hybrids like A/D (Bgl) for in vitro and in vivo studies in mice. The frequent administration of the maximal dose could compare with the optimal protocol for augmenting immunologic reactivity to determine their relative therapeutic efficacy. The establishment of the parameters for optimal anti-tumor effects in mouse tumor models would be expected to be helpful for the rational design of clinical trials.

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Augmentation of Natural Killer Activity by Hybrid Interferons

Effects of recombinant and hybrid recombinant human leukocyte interferons on cytotoxic activity of natural killer cells.
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