The Influence of Interferon Alpha and Gamma, Singly or in Combination on Human Natural Cell Mediated Cytotoxicity

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The influence of interferon alpha and gamma alone or in combination on the augmentation of human natural cytotoxicity was studied. Treatment of peripheral blood lymphocytes with IFN-α led to a rapid augmentation of NK activity, in contrast to IFN-γ where target cell killing was observed only following 18 hrs exposure of lymphocytes to IFN-γ. The results of the single cell assay paralleled those obtained using the Chromium release test, but neither interferon type caused an increase in the number of target binding lymphocytes. The combined effect of IFN-α and IFN-γ in stimulating human natural cytotoxicity demonstrated individual lymphocyte responses to be variable. Exposure of lymphocytes to IFN-α and IFN-γ for 18 hrs prior to assay for cytotoxicity usually decreased the level of cytotoxicity compared with control values, whereas other treatment regimes gave an additive and sometimes synergistic effect. Only treatment with IFN-α for 18 hrs and IFN-γ for one hr produced a synergistic response in the majority of individuals tested. We conclude from this study that individual responses to IFN-α and IFN-γ alone or in combination are variable and dependent upon timing of exposure of lymphocytes to individual interferon types, and possibly reflects the donor status at the time of sampling.

KEY WORDS: NIC; human; interferon; activation; cytotoxicity.

INTRODUCTION

Interferons have been shown to mediate a wide range of biological activities (Dolei et al., 1983; Tsujimoto and Vilcek, 1986; Wallack, 1983), and evidence suggests that their antitumour effects may be attributed to a direct antiproliferative effect (Bulkwill et al., 1982; Fresa and Murasko, 1986) and indirectly by modulation of host immune defence mechanisms (Fresa and Murasko, 1986; Reid et al., 1981). In view of the proposed role of NK cells in immunesurveillance (Wiltrout et al., 1985), modulation of NK cell activity by interferons is potentially important in controlling tumour growth.
Augmentation of NK cytotoxicity by interferon is well documented, but few reports have directly compared the effects of the individual interferon types on NK cell mediated killing. Neither Perrussia et al. (1980) nor Senik et al. (1980) observed any differences between the efficacy of the interferons they used to increase NK cell activity. In contrast, Weigent et al. (1982) reported IFN-γ to be both more effective and to act more rapidly than other IFN-α or IFN-β in augmenting NK cell cytotoxicity. Here we report that while both interferon types are capable of increasing the cytotoxic capacity of human NK cells, the kinetics of augmentation of IFN-α versus IFN-γ is distinct. Furthermore, combinations of IFN-α and IFN-γ can produce a synergistic response, which is variable between individuals and also depends upon the treatment regime used.

MATERIALS AND METHODS

Interferons

Human leukocyte IFN (IFN-α) (PIF 7901), produced in peripheral blood leukocytes stimulated with Sendai virus and partially purified to a specific activity (SA) of $1 \times 10^6$ U/mg protein was a gift from Kari Cantell, State Serum Institute (Helsinki, Finland). Human IFN-γ, produced in peripheral blood leukocytes induced with A23187 and mezerine (SA > $1 \times 10^6$ U/mg protein) was obtained from Meloy Laboratories (Springfield, VA 22151, USA). The antiviral activity of IFN preparations was determined in WISH cells challenged with encephalomyocarditis virus using modifications of previously described methods (Armstrong, 1971). Interferon titres are expressed in terms of appropriate reference standard for human interferons distributed by the Research Resources Branch, National Institute of Allergy and Infectious Diseases (Bethesda, MD, USA) (IFN-α, Gi-023-901-527; IFN-γ, Gg23-901-530).

Effector Lymphocytes

Fresh peripheral blood lymphocytes (PBL) were obtained from heparinised blood by normal volunteers by density gradient centrifugation over Lymphoprep (Nycomed, Birmingham, England) and passaged over nylon wool to remove adherent cells (Rees et al., 1975) and 1 ml cultures of non-adherent lymphocytes ($4 \times 10^6$ cells/ml) incubated in 24 well culture plates (plates from Falcon Becton Dickinson) with 100 µ of either IFN-α or IFN-γ. Precise details of culture conditions are given for individual experiments in the Results Section.

Target Cells and Cytotoxicity Assay

The NK sensitive cell line K562 was maintained as a suspension culture in RPMI-1640 supplemented with 10% Newborn Calf Serum and antibiotics. Cultures in log growth phase only were used as target cells in the cytotoxicity assay. The colorectal carcinoma cell line SW742, which is resistant to spontaneous
human NK cytolysis, was obtained from Dr W. McCombs, Scott and White Clinic, Temple, Texas, and was grown as a monolayer in RPMI-1640 supplemented with 10% Foetal Calf Serum and antibiotics. Both cell lines were mycoplasma free.

Chromium release assays were performed as previously described (Rees et al., 1987). Briefly $1 \times 10^4 \ 51\text{Cr}-$labelled target cells in 0.2 ml of RPMI-FCS were incubated with an equal volume of effector cells in round bottomed microtest plates (Falcon Microtest III, Flexible Assay Plates, Becton Dickinson) at various effector:target ratios. Following incubation at $37^\circ\text{C}$ in a humidified incubator (5%-CO$_2$ 95%-air) for 4 hours, 0.1 ml of the supernatant (SN) was removed into separate wells. The plate was then dried and the individual wells counted for radioactivity in a gamma spectrophotometer. All tests were carried out in triplicate and the percentage $51\text{Cr}$-release and percentage cytotoxicity calculated by the following formulae.

\[
\text{Percentage } 51\text{Cr}-\text{release} = \frac{\frac{1}{2}(\text{SN}) \times 2}{\frac{1}{2}(\text{SN}) + \text{cell pellet}} \times 100
\]

\[
\text{Percentage cytotoxicity} = \frac{\text{test } 51\text{CR release} - \text{spontaneous } 51\text{Cr-release}}{100 - \text{spontaneous } 51\text{Cr-release}} \times 100
\]

The spontaneous $51\text{Cr}$-release in the presence of medium alone ranged from 5–10%. Statistical analysis was performed by the students T test where appropriate.

**Single Cell Assay**

A modification of the single cell cytotoxicity assay in agarose previously described (Silva et al., 1980) was employed. A solution of 1% agarose (Oxoid, Basingstoke, England) supplemented with 10% foetal calf serum was maintained in a 42°C water bath. Equal numbers of lymphocytes and targets were suspended in 2 ml of medium in 16 × 100 mm conical bottomed tubes (Sterilin, Middlesex, England) centrifuged at 450 g for 5 minutes at room temperature, and incubated for 10 minutes in a 37°C water bath. All but 0.2 ml of medium was removed and the cell pellet gently resuspended. 2 ml of agarose solution was added and 0.5 ml of the suspended cells were removed and spread onto precoated 60 mm petri dishes (Sterilin). Once the agar had solidified 2 ml of medium was added to each plate which was then incubated at 37°C under 5% CO$_2$. After 2, 4 and 6 hours the plates were rinsed with PBS and stained with trypan blue. Conjugate formation and target killing was assessed immediately using a microscope. Target cell death in control plates without effector cells was always less than 2%. The results are expressed in terms of the total number of lymphocytes killing the target (n) calculated by the following formula:

\[
n = \frac{\text{number of bound lymphocytes} \times \text{number of lymphocytes killing target}}{100}
\]
RESULTS

The effect of IFN-α and IFN-γ on the Augmentation of NK Cell Mediated Cytotoxicity

IFN-α or IFN-γ was added to lymphocyte cultures at 1, 2 or 18 hours before harvesting the cells and assessing NK-cell activity against K562 and SW742 targets. The results of these experiments are summarised in Fig. 1, and represent the mean of 16 experiments for IFN-α and 14 experiments for IFN-γ. Our initial studies showed that killing of SW742 targets was insensitive to spontaneous NK attack but a reliable indicator of activated human NK cells; K562 in contrast, is highly susceptible to spontaneous NK cell cytotoxicity.

IFN-α significantly augmented NK-cell activity (SW742 targets) at 1, 2 and 18 hours (P < 0.001), while IFN-γ augmented NK-cell mediated cytotoxicity only following 18 hours stimulation (P < 0.001). In addition, the lymphocyte response to IFN-γ at 18 hours was significantly lower (P < 0.005) than the response elicited by IFN-α at comparable antiviral unit concentrations. Only 9 out of 16 individuals responded to IFN-γ, while all PBL preparations were stimulated by IFN-α. Similar results have been obtained using human recombinant IFN-α (results not given).

Kinetics of Augmentation of NKCC by IFN-α and IFN-γ

The kinetics of augmentation by IFN-α and IFN-γ was investigated and the results are shown in Fig. 2. IFN-α induced a rapid increase in NK cell activity against SW742 which peaked 4 and 6 hours after stimulation and declined to approximately 50% of the maximum stimulation, but remained significantly higher than that of the control (P < 0.001) during the subsequent 40 hour period. Although the response to IFN-γ was significant 18 hours after stimulation

![Fig. 1](image-url)  
**Fig. 1.** Effect of IFN-α and IFN-γ on natural cell mediated cytotoxicity. Lymphocytes were cultured for 18, 2 and 1 hour in the presence of 100μg of IFN-α (□) or IFN-γ (■). Cytotoxicity was assessed in a 4 hour chromium release assay against SW 742 and K562 target cells. The results represent the mean of 16 and 14 experiments for IFN-α and IFN-γ respectively. Bar lines indicate standard deviation. * Denotes a significant augmentation above control (□) levels of cytotoxicity P < 0.001.
NK Activation by IFN-α and IFN-γ

Fig. 2. The kinetics of augmentation of natural cell mediated cytotoxicity by IFN-α and IFN-γ. Lymphocytes were cultured with 100μ of IFN-α or IFN-γ for the appropriate time before assessing cytotoxicity against the target cell line SW742.

(P < 0.001), maximum augmentation was achieved after 40 hours or more of culture (results confirmed in four separate experiments).

Single Cell Assay Analysis

Effector lymphocytes were treated with either IFN-α or IFN-γ for 18 hours, 2 hours or 1 hour and their ability to form conjugates and to mediate killing in a single cell assay was analysed. Treatment of lymphocytes with either IFN-α or IFN-γ did not result in a change in the percentage of conjugates formed with either K562 or SW742 targets; however, the percentage binding of effector cells to SW742 cells was consistently lower than with K562 (results not given).

Lymphocytes were further analysed for their ability to mediate killing in single cell assays performed in agarose against both SW742 and K562 targets, and Fig. 3 shows the results of a representative experiment using SW742 target cells. Effector cells were treated with IFN-α or IFN-γ for 1, 2 or 18 hours and assayed for cytotoxicity using a single cell assay. Using IFN-α, all three treatments resulted in increased kinetics of kill, together with an increase of at least 50 percent in the number of bound cells capable of lysing the target (Fig. 3). In agreement with the ⁵¹Cr-release data, PBL’s required exposure to IFN-γ for 18 hours to demonstrate enhanced single cell killing. Treatments which did not boost NK cell activity in the chromium release assay failed to show a significant difference from control cytolysis in the single assay (confirmed in separate experiments and using either SW742 or K562 target cells).
Fig. 3. Single cell analysis of lymphocytes treated with interferon. Lymphocytes were treated with IFN-α (○—○) or IFN-γ (□—□) for 18, 2 or 1 hour and analysed together with control cells (●—●—●) in a single cell assay against SW742 target cells. One of three experiments performed.

Effect of Combined IFN-α and IFN-γ on Cytotoxicity

Effector lymphocytes were incubated with either IFN-α or IFN-γ for 18 hours with interferon of the opposite type added to the culture 18 hours, 2 hours or 1 hour prior to assay. Only one treatment (18 hours IFN-α + 1 hour IFN-γ) appeared to be synergistic in influencing cytotoxicity (P < 0.001). Of the remaining four treatments, three were additive with no difference being observed between the expected and observed values, and one (18 hours IFN-α + 18 hours IFN-γ) causing a reduced cytotoxicity (P < 0.005) compared with the expected value. Analysis of individual responses revealed that within the groups showing an overall additive response, approximately half the individuals demonstrated a synergistic response to the two interferons. These findings are summarised in Tables 1 and 2.
Table 1. Analysis of the group data of augmentation of natural cytotoxicity by combinations of IFN-α and IFN-γ

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of experiments</th>
<th>Mean expected cytotoxicity</th>
<th>Mean observed cytotoxicity</th>
<th>Fold potentiation</th>
<th>t-Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 hours IFN-α + 18 hours IFN-γ</td>
<td>6</td>
<td>38 (15)</td>
<td>30 (7)</td>
<td>0.8</td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td>18 hours IFN-α + 2 hours IFN-γ</td>
<td>9</td>
<td>28 (15)</td>
<td>35 (16)</td>
<td>1.25</td>
<td>NS</td>
</tr>
<tr>
<td>18 hours IFN-α + 1 hour IFN-γ</td>
<td>9</td>
<td>28 (17)</td>
<td>31 (17)</td>
<td>1.11</td>
<td>NS</td>
</tr>
<tr>
<td>18 hours IFN-γ + 2 hours IFN-α</td>
<td>9</td>
<td>32 (18)</td>
<td>37 (18)</td>
<td>1.15</td>
<td>NS</td>
</tr>
<tr>
<td>18 hours IFN-γ + 1 hour IFN-α</td>
<td>9</td>
<td>23 (16)</td>
<td>33 (17)</td>
<td>1.43</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

a Non-adherent lymphocytes treated with 100 μl of either IFN-α or IFN-γ for 18 hours with 100 μl of interferon of the opposite type added 18 hours, 2 hours or 1 hour prior to harvesting the cells.

b Sum of the cytotoxicity of the individual treatments.

c Mean observed cytotoxicity—mean expected cytotoxicity.

d Mean cytotoxicity ±SD.

DISCUSSION

The present study was undertaken to compare the effect of IFN-α and IFN-γ, individually and in combination on NK cell mediated cytotoxicity in vitro. The kinetics of augmentation were distinct for each interferon type. Whereas IFN-α increased NK cell cytotoxicity following culture with effector cells for 1–2 hours, significant augmentation of NK cell function by IFN-γ occurred only after treatment of lymphocytes in excess of 18 hours. Braakman et al. (1986) described a similar pattern of augmentation with IFN-γ, contrasting with those of Weigent et al. (1982), who used an 18 hour assay with L-cells as targets to measure natural cytotoxicity, which may be inappropriate for assessing augmentation of NK cell activity. The response of NK cells to recombinant IFN-α appeared to be similar, both in magnitude and kinetics, to the response induced by the naturally produced IFN-α. This contrasts with the disparate response of lymphocytes to

Table 2. Summary of the individual responses to treatment with combinations of IFN-α and IFN-γ

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of individual experiments giving a synergistic response</th>
<th>Number of individual experiments giving an additive response</th>
<th>Number of individual experiments showing decreased cytotoxicity</th>
<th>Overall group response</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 hours IFN-α + 18 hours IFN-γ</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>Decrease in cytotoxicity</td>
</tr>
<tr>
<td>18 hours IFN-α + 2 hours IFN-γ</td>
<td>9</td>
<td>4</td>
<td>5</td>
<td>Additive</td>
</tr>
<tr>
<td>18 hours IFN-α + 1 hour IFN-γ</td>
<td>9</td>
<td>4</td>
<td>5</td>
<td>Additive</td>
</tr>
<tr>
<td>18 hours IFN-γ + 2 hours IFN-α</td>
<td>9</td>
<td>5</td>
<td>4</td>
<td>Additive</td>
</tr>
<tr>
<td>18 hours IFN-α + 1 hour IFN-γ</td>
<td>9</td>
<td>8</td>
<td>1</td>
<td>Synergistic</td>
</tr>
</tbody>
</table>

a Non-adherent lymphocytes treated with 100 μl of either IFN-α or IFN-γ for 18 hours; 100 μl of interferon of the opposite type added 18 hours, 2 hours or 1 hour prior to harvesting the cells.
natural and recombinant IFN-γ. In the present study recombinant IFN-γ was an ineffective inducer of augmentation of natural cytotoxicity, a result which is again in contradiction with those of Weigent et al. (1982). Several cell types have however shown a reduced ability to respond to recombinant IFN-γ compared with the natural molecule (Littman et al., 1987), and such quantitative differences may reflect the lack of glycosylation on the recombinant IFN-γ molecule. The poor performance of recombinant IFN-γ in in vitro assays must raise questions as to the efficacy of its use in clinical trials. This is not the case with IFN-α, where the naturally occurring molecule is not glycosylated.

The results presented here using single cell analysis of cytotoxicity show that whilst the number of conjugates remains constant for both interferon treated and control cultures, there is an increase in the number of bound effectors capable of killing their targets. The increase in the kinetics of killing mediated by interferon treated cells, was correlated with the observed increase in NK cell activity assayed by chromium release, and this is comparable with those previously reported by Silva et al. (1980). The resistance of SW742 targets to spontaneous NK cell mediated killing may depend on factors which prevent delivery of the "lethal hit" leading to target lysis, which is overcome once NK effector cells have been activated with IFN. In view of the results presented here it can be inferred that K562 cells are not the most appropriate target to use to assess activated NK cells, a point raised by previous authors (Van der Griend et al., 1986). The use of solid tumour cell targets, such as human colon carcinoma and melanoma cell lines, may offer a more appropriate in vitro system for studying NK cell activation (Rees et al., 1987).

Synergistic interaction between IFN-α and IFN-γ has previously been described for their antiviral activity (Schwartz et al., 1984) and antitumour action (Fleischmann et al., 1980). In the present study combinations of the two interferons resulted in synergism in augmenting NK cell cytotoxicity, although the response was variable between individuals and was dependent on the treatment regime used. This may be a consequence of interferon receptor modulation; thus, the expression of Type I interferon receptors are influenced by IFN-γ, expression of Type I receptors increasing for the first ten hours following the exposure to IFN-γ, followed by a steady decline in IFN-α binding over the subsequent 24 hour period (Hannigan et al., 1984). Such a phenomena may, in part, explain the strong synergistic response in 8 out of 9 individuals following treatment for 18 hours with IFN-α and one hour with IFN-γ. In contrast, down regulation of type I receptors by IFN-α is rapid and monophasic (Branca and Baglioni, 1982) and may contribute to the variable responses observed with other combined treatment protocols.

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REFERENCES