Human Alpha and Gamma Interferon Analogs in Rabbits with Herpetic Keratitis

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Complete gene synthesis methods have been used to construct analogs of human interferons (IFNs): these include a consensus of the known human IFN-αs, designated IFN-αCon, and a variant of human IFN-γ, designated IFN-γ4A. These interferons, in purified form, were used topically against herpes simplex virus type 1 (HSV-1) induced ocular keratitis in rabbits. Eyes pretreated with IFN-αCon had decreased signs of infection and a lower incidence of HSV-1 positive trigeminal ganglia (3 of 14 positive) compared to the placebo treated (10 of 14 positive). IFN-αCon was as effective as natural IFN-α subtypes on a units basis, despite the very high specific activity of this analog. IFN-γ4A used under similar conditions do not result in beneficial effects with treatments beginning 24 or 48 hr before or after virus inoculation. Rabbits with confirmed latent HSV infection were treated topically with IFN-αCon (10^6 units per eye each day) either before or before and after attempts to intentionally reactivate the infection by bilateral iontophoresis of 6-hydroxydopamine plus topical epinephrine treatment of the corneas. These IFN-αCon treatment regimens along with intentional reactivation during latency did not: (1) lessen the frequency of inducible ocular shedding episodes; (2) alter the mean time of 3–5 days between attempts to reactivate latent infection and the appearance of HSV in tears; or (3) significantly change the incidence of HSV-positive trigeminal ganglia (83–100% HSV positive). Invest Ophthalmol Vis Sci 26:1244–1251, 1985

Human interferons (IFNs) are classified on the basis of physicochemical criteria into three groups, alpha (IFN-α), beta (IFN-β), and gamma (IFN-γ) interferons. The primary structure of these materials has been elucidated by recombinant DNA methods. Human IFN-β and IFN-γ show little or no variations in sequence and occur as single gene copies in the genome. In contrast, the number of IFN-α genes in the human genome has been estimated at 16 to 20,¹ consistent with the dozen or more molecular species observed in highly purified natural IFN-α preparations.²

Human IFN-α preparations have shown antiviral activity to various degrees in a variety of mammalian cell cultures,³ while IFN-β shows more restricted species specificity. The species specificity of IFN-γ appears to more closely resemble IFN-β than IFN-α.⁴ These interferons have pronounced antiviral activity in rabbit cell cultures, sometimes exceeding the activity observed in human cell cultures. HSV-1 induced ocular keratitis in the rabbit has proved to be a useful model for the disease in man and particularly useful for human interferons because they are effective in the rabbit model. Significant activity has been observed in vivo with various human IFN-α preparations, even with IFN-α subtypes that have very low antiviral activity in rabbit cell cultures.⁵ Human IFN-β is ineffective against HSV-1 infection of the rabbit eye, despite significant activity in rabbit cell cultures.⁶ The in vivo mechanism of action of interferons against HSV is thus indirect,⁷,⁸ as observed for several other viral infections.⁹

Analogs of human IFN-α and IFN-γ have been obtained using complete gene synthesis methods.¹⁰ The known human IFN-α sequences were used as a basis for devising an analog that is a consensus of the various IFN-α subtypes. This analog is called alpha consensus interferon, or IFN-αCon. It appears to incorporate properties of various IFN-α subtypes and has a specific antiviral activity in human cells 10 to 20 times greater than that of any known IFN-α.¹⁰ A series of IFN-γ analogs has been constructed in a
similar manner, each involving one or a few amino acid substitutions or deletions when compared with the sequence deduced by recombinant DNA methods. One of these analogs, lacking the first three residues predicted from the gene sequence, has particularly desirable properties; this material is called gamma 4A or IFN-γ4A. Although originally conceived as an analog, this interferon now seems to correspond to the sequence of natural IFN-γ, which also lacks three residues from the predicted sequence at the N-terminus.

In the present studies we assessed the efficacy of IFN-αCon, and IFN-γ4A against HSV-1 induced ocular keratitis in the rabbit. Of particular interest was the relationship of the high antiviral activity of IFN-αCon in cell cultures to in vivo efficacy and assessment of the activity of IFN-γ in the rabbit model system.

Materials and Methods

Interferon Preparations

A gene coding for a consensus of the known IFN-α subtypes was constructed by the phosphite chemistry method and expressed under trp control in Escherichia coli. This interferon, IFN-αCon1, differs in 10 residues from one of the known natural subtypes, namely IFN-αF, as defined elsewhere. Similarly, a gene coding for human IFN-γ but lacking the first three predicted amino acid residues was constructed and expressed in E. coli. This interferon is termed IFN-γ4A and can be readily formulated and stored because it lacks cysteine residues which tend to cause intermolecular bridges. These materials are purified to homogeneity as assessed by polyacrylamide gel electrophoresis. The specific activity of IFN-αCon1 was $10^9$ U/mg protein and for IFN-γ4A was $2 \times 10^7$ U/mg protein. All interferon titrations were by cytopathic inhibition assays on HeLa cells infected with encephalomyocarditis virus calibrated against the NIH IFN-α standard (GO23-901-527) and the IFN-γ standard (Gg23-903-530). The IFN-α standard was found to be useful for IFN-γ4A because in the assay used this was shown to produce a parallel dose response curve, as previously demonstrated for other cloned IFN-αs and IFN-γ.

Ocular Inoculation with HSV-1

New Zealand white rabbits, weighing 2–3 kg, were housed throughout this study conforming to the ARVO Resolution on the Use of Animals in Research, and were examined for preinoculation ocular abnormalities. Stock laboratory HSV-1 (McKrae strain) was prepared and titrated in rabbit kidney (RK) cells and stored at −70°C. On day 0, HSV-1 (10⁵ PFU/eye) was placed in the lower cul-de-sac of both eyes and the lids were closed and gently massaged for 30 sec.

Reactivation of Latent HSV-1

Rabbits were confirmed to have a primary HSV-1 infection by isolation of virus on the third day after inoculation. At 28 days after inoculation, rabbits were intentionally stimulated to induce shedding of virus using a modification of the Shimomura iontophoresis procedure. Briefly, anesthetized rabbits were fitted with an eye cup containing 0.1% 6-hydroxydopamine. The anode (+) terminal was placed in the eye cup and the cathode (−) terminal attached to the ear. A direct current (0.6 mA) was applied for 6–8 min. This procedure was performed on each eye and was followed by topical treatment with 2% epinephrine. Only rabbits in which HSV-1 was isolated from tear film samples following stimulation in this manner and verified by microneutralization studies with known reference antiserum were employed in the subsequent reactivation studies. In our laboratory, this method for reactivation of latent virus results in virus being shed into the tears of at least one eye within a week in approximately 75% of the latently infected rabbits. When the procedure is repeated on the same animals, the rate of induced reactivation declines.

Topical Treatment

For the initial acute infection study, therapy was initiated at either 24 hr before or 4 hr after virus inoculation. Rabbits in this study were divided randomly into different treatment groups (6 rabbits/group) and received topical $10^6$, $10^5$, or $10^4$ U/eye/day of IFN-αCon1 diluted in saline and stored at 0–4°C until use; four 50 μl/dose were given for 14 consecutive days. The saline placebo treatment was given on the same schedule. For the second acute infection study, comparison of topical IFN-αCon1 and IFN-γ4A, nine rabbits received $10^6$ U/eye/day of IFN-αCon1. Three rabbits received $10^6$ U/eye/day of IFN-γ4A and nine rabbits received a saline placebo. Treatment was carried out four times a day (50 μl/dose) for 7 consecutive days.

Six rabbits with latent HSV-1 infection were re-stimulated starting on day 91 postinfection with therapy consisting of $10^6$ U/eye/day of IFN-αCon1, from day 92 to 100. Five other latently infected rabbits were treated similarly with IFN-αCon1, from...
Fig. 1. Degree of severity of corneal epithelial involvement (a) and conjunctivitis (b) observed in rabbits following inoculation with 10^5 PFU per eye of HSV-1 (McKrae). Beginning 24 hr before or 4 hr after inoculation eyes were treated topically 4 times/day with either IFN-αCon, (10^4-10^6 U/eye/day) or placebo (saline). Treatment started at 24 hr before inoculation was continued for 7 consecutive days while treatments started at 4 hr postinoculation continued for 14 days. When corneal lesions were first detectable on day 3, eyes were examined for pathologic changes and then re-examined on alternate days during therapy. IFN-αCon, (10^6 U/eye/day) 24 hr before inoculation (●); IFN-αCon, (10^6 U/eye/day) beginning 4 hr after inoculation (▲); IFN-αCon, (10^5 U/eye/day) beginning 4 hr after inoculation (●); IFN-αCon, (10^4 U/eye/day) beginning 4 hr after inoculation (□); placebo beginning 4 hr after inoculation (○).

day 91 to 100 and restimulation initiated on day 95. As a control group, five other latently infected rabbits underwent restimulation on day 91 and received placebo (saline) treatment from day 92 to 100.

Ocular Examination

Eyes with acute infection were examined daily without prior knowledge of the treatment involved by the same observer using a 0 (normal) to 4+ (most severe) grading system for each of the following: corneal epithelial involvement, conjunctivitis, iritis, and corneal clouding. The average score for each group was calculated from day 0 to 14.

One-way analysis of variance was performed for each clinical parameter at each time point to detect differences due to treatment regimens. If overall significant differences (P < 0.05) were found, the Newman-Keuls multiple comparison method was used to determine which groups were significantly different from the control or other treatment groups.

Isolation of HSV from Tear Film and Tissue

Tear film specimens for virus isolation were collected each day before the first treatment by washing the globe with saline containing penicillin (100 U/ml)–streptomycin (100 mg/ml, Gibco; Grand Island, NY). The washings were inoculated onto secondary rabbit kidney cells maintained at 37°C and examined for cytopathic effects (CPE) for 7 days.

Trigeminal ganglia were removed aseptically, minced and cocultivated with secondary RK cells. These cocultivated cultures were incubated at 37°C and examined microscopically three times a week for up to 4 wk for CPE. The identity of all suspicious isolates was confirmed as HSV by microneutralization test of the culture supernatant with reference antiHSV serum.

Results

Topical IFN-αCon, Treatment for Acute Ocular HSV Infection

The degree of severity for corneal epithelial involvement was inversely proportional to the dose of IFN-αCon, employed for therapy (Fig. 1a). When IFN-αCon, treatment (10^6 U/eye/day) was initiated 24 hr before virus inoculation and continued for 7 days, the corneal epithelial involvement was the least severe, but not significantly less (P > 0.05) than when the same treatment (10^6 U/eye/day) was begun at 4 hr after inoculation and continued for 14 days. However, signs in these two groups were significantly less severe (P < 0.05) than other treatment groups (ie, 10^5, 10^4 U/eye/day or placebo), in which corneal epithelial involvement was most intense on day 5. There was no significant difference (P > 0.05) in the
severity of corneal epithelial involvement for groups receiving $10^5$ or $10^4$ U/eye/day, or placebo.

The extent of conjunctivitis observed in these same rabbits was similar in that treatment with IFN-αCon1 (10^6 U/eye/day) beginning at either 24 hr before or 4 hr after inoculation resulted in significantly less ($P < 0.05$) conjunctivitis during days 4 to 6 (Fig. 1b). No difference in conjunctivitis was observed for groups treated with $10^5$ or $10^4$ U/eye/day IFN-αCon1 compared with the placebo-treated control group. The severity of iritis and corneal clouding in these animals (data not shown) was either undetectable (eg, $10^6$ U/eye/day of IFN-αCon1 initiated at 24 hr preinoculation) or only minimal, for the other four treatment groups and none of the group mean values were significantly different (data not shown, $P > 0.05$).

When IFN-αCon1 therapy was initiated at 4 hr postinfection, each treatment group had one or two deaths occurring between days 12 and 25 (Table 1). However, no deaths (0/7) were observed in those rabbits that began receiving $10^6$ U/eye/day at 24 hr before virus inoculation. When we examined the trigeminal ganglia from these same animals for virus, the isolation of HSV was significantly less ($P < 0.01$) following IFN-αCon1 treatment (except for the $10^4$ U/eye/day group) when compared to the placebo group (Table 1).

Comparison of Effect of Topical IFN-αCon1 and IFN-γ4A Therapy

The degree of severity for corneal epithelial involvement was significantly reduced ($P < .005-.018$) in HSV-1 infected eyes treated with $10^6$ U/eye/day of IFN-αCon1 during peak involvement on days 3 to 6 (Fig. 2a) as observed in previous studies. However, no differences were obvious between the IFN-γ4A and placebo-treated groups throughout the study, except for day 13 when no corneal epithelial involvement was observed in the placebo group (Fig. 2a).

Conjunctivitis was significantly less ($P < 0.001-0.006$) in eyes receiving IFN-αCon1 from days 3 to 8 compared to IFN-γ4A and placebo-treated eyes (Fig. 2b). No statistically significant differences were detected between the IFN-γ4A and placebo-treated control group even when therapy was initiated at 48 or 24 hr before virus inoculation (data not shown).

The appearance of iritis and corneal clouding was delayed and the severity was significantly lower ($P < 0.001-0.027$) in the IFN-αCon1 treated eyes. For example, the highest severity scores of iritis and clouding were recorded on day 12 for the IFN-αCon1 group and on days 7–8 for IFN-γ4A and placebo groups (Figs. 2c, 2d).

No change was noted in the duration of HSV-1 shedding into the tear film of rabbits during topical IFN-αCon1 or IFN-γ4A treatment for acute herpetic keratitis (Table 2). Fifty percent or more of the eyes treated with IFN-αCon1 were HSV negative 10 to 12 days after infection. When ocular culturing for virus isolation was stopped on day 13, eyes were negative for HSV. Statistically, there was no difference between any of the treatment groups for duration of virus shedding.

Table 1. Mortality and HSV isolation from trigeminal ganglia (TG) of rabbits treated for acute ocular HSV infection with topical IFN-αCon1

<table>
<thead>
<tr>
<th>No. of rabbits</th>
<th>IFN-αCon1 treatment (U/eye/day)</th>
<th>No. of deaths (days until death)</th>
<th>HSV positive TG (Total TG tested)</th>
<th>(P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>Placebo</td>
<td>2 (15, 15)</td>
<td>10/12</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>$10^6$</td>
<td>2 (15, 25)</td>
<td>3/10 (P &lt; 0.01)</td>
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<tr>
<td>7</td>
<td>$10^5$</td>
<td>1 (15)</td>
<td>3/12 (P &lt; 0.01)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>$10^4$</td>
<td>2 (12, 14)</td>
<td>6/10 (NS)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>$10^3$</td>
<td>0</td>
<td>3/14 (P &lt; 0.01)</td>
<td></td>
</tr>
</tbody>
</table>

NS: not significant.
* Bilateral therapy was initiated at 24 hr before inoculation (IFN-αCon1, given 4X/day [9 am, 11 am, 2 pm and 4 pm] and was continued for 7 days). All other treatment regimens were the same except therapy began at 4 hr postinoculation and continued for 14 days.
† Chi-square analyses were performed to test whether the rates of virus isolation were different across the treatment groups ($x^2 = 14.07$, df = 4, $P < 0.01$ and $x^2 = 1.04$, df = 1, NS).

Topical IFN-αCon1 Treatment and Intentionally Reactivated Latent HSV-1 Infection

Data for shedding of HSV into tears from latently infected rabbits following combined intentional reactivation and topical IFN-αCon1 therapy are presented in Table 3. Three of six rabbits undergoing intentional reactivation beginning on day 91 and treated topically with IFN-αCon1 (10^6 U/eye/day) shed virus into tear film samples during therapy. Ten trigeminal ganglia of the 12 in six rabbits were HSV-positive. Three of five rabbits shed virus when IFN-αCon1 was given 4 days before and 5 days after reactivation on day 95. All 10 trigeminal ganglia of these five rabbits were HSV-positive. Two of five rabbits shed virus following an identical regimen with placebo and nine of 10 of the trigeminal ganglia were HSV-positive.

Discussion

Although IFNs show some species-specificity, significant biological activity can be observed in heter-
Table 2. Duration of HSV-1 shedding into tear film* during topical IFN-αCon1 and IFN-γ4A treatment for acute herpetic keratitis

<table>
<thead>
<tr>
<th>No. of rabbits</th>
<th>Treatment†</th>
<th>Days post-inoculation</th>
<th>Days post-inoculation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>until ≥50% eyes negative</td>
<td>until 100% eyes negative</td>
</tr>
<tr>
<td>9</td>
<td>Placebo</td>
<td>11</td>
<td>&gt;13</td>
</tr>
<tr>
<td>9</td>
<td>IFN-αCon1</td>
<td>12</td>
<td>&gt;13</td>
</tr>
<tr>
<td>3</td>
<td>IFN-γ4A</td>
<td>10</td>
<td>13</td>
</tr>
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</table>

* Eye washes were collected each morning before the first daily treatment and inoculated onto secondary RK cell cultures. Inoculated cultures were examined microscopically for cytopathic effect for 7 days.
† Bilateral topical treatment was administered 4 times/day (9 am, 11 am, 2 pm, and 4 pm).

Fig. 2. Degree of severity of corneal epithelial involvement (a), conjunctivitis (b), iritis (c) and corneal clouding (d) observed in rabbits (same experimental conditions as described for Figure 1 except when topical treatment was for 7 days and consisted of 10⁶ U/eye/day of IFN-αCon1 or IFN-γ4A). Placebo (O); IFN-γ4A (●); IFN-αCon1 (X).
Table 3. Ocular HSV shedding and virus positive ganglia from latently infected rabbits treated with IFN-αCon

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rabbit no.</th>
<th>Eye</th>
<th>Days of treatment</th>
<th>Number positive trigeminal ganglia</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-αCon₁ (after stimulation)</td>
<td>1</td>
<td>OD</td>
<td>91, 92, 93, 94</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>OD</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>OD</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>OD</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>OD</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>OD</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Placebo (after stimulation)</td>
<td>3</td>
<td>OD</td>
<td>91, 92, 93, 94</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>OD</td>
<td>+</td>
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<tr>
<td></td>
<td>20</td>
<td>OD</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>OD</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>OD</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>OD</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>OD</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>OD</td>
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<td></td>
<td>32</td>
<td>OD</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>33</td>
<td>OD</td>
<td>+</td>
<td></td>
</tr>
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</table>

OD: Reactivation; +: HSV positive tear film.

doses, on a units basis, comparable with those of Smolin et al⁵ and Grabner et al⁶ and higher doses were not used. However, our results do indicate that longer treatment periods (14 days) are not significantly better than 7 days. The fact that Sanitato et al²² found that high doses of a natural IFN-α preparation are effective in rabbits against HSV-1 indicates that the presence of IFN-α subtypes in natural preparations do not lose antiviral activity at high doses.

The absence of significant effects with IFN-γ₄₄ against HSV-induced ocular keratitis in the rabbit may be related to negligible antiviral activity of this interferon in rabbit cell cultures. However, there are cases where IFNs without antiviral activity in cell cultures are effective in vivo⁹ and a few amino acid substitutions can have dramatic effects on pharmacologic effects of interferons.²³ However, the rabbit eye model seems to be unsuitable for assessing the utility of human IFN-γ₄₄ against HSV keratitis. It remains to be seen whether IFN-γ₄₄ is useful in human clinical herpetic disease.

The present studies provide evidence that novel recombinant DNA derived IFN-α analogs can be tested successfully in heterologous systems. Antiviral activity was demonstrated in the rabbit eye; however, the really important question of whether this particular IFN-α (IFN-αCon₁) is efficacious in the human clinical situation for herpes infections remains unanswered. Recent studies have suggested that combined therapies involving antiviral chemotherapeutic agents plus IFN-α are more effective than a monotherapy.²⁴-²⁸ Early treatment with high concentrations of IFN-αCon₁ was effective in reducing the severity of acute herpetic keratitis and in reducing
the incidence of HSV infection of the trigeminal ganglia, which are known to be a reservoir for the latent virus.29 However, this same potent dose (10⁶ IFN-αCon1 U/eye/day) did not have demonstrable influence on intentionally reactivated latent HSV infections. Absence of effects of interferon on reactivation of latent HSV-1 infections have been observed in other models, but this may not be the case in human disease. Pazin et al10 have observed clinical efficacy with IFN-α treatments given post surgically to patients susceptible to HSV after sectioning of the trigeminal nerve. It will be interesting to assess the immunologic effects of IFN-αCon1, IFN-γ44a and other IFNs and relate these properties to mechanisms that may be shown to be of importance in development and limitation of clinical HSV-1 infections, both acute and latent.

Although the mechanisms of action of IFNs against HSV ocular keratitis appear to be indirect, they may be correlated with antiviral activity in cell cultures, at least for IFN-αs. The IFN-αD subtype is more effective than IFN-αA in rabbit cells and against ocular infection.6,23 and in the present studies IFN-αCon1 was used at doses of comparable antiviral units to those used in earlier studies. However, the amount by weight of IFN-αCon1 was much lower because of the high specific activity of this IFN. The high specific activity of IFN-αCon1 could be clinically useful if side effects of IFN-αs are related to the amount of material used rather than the antiviral potency of the material administered. When IFN-αCon1 was compared with IFN-γ44a on an equal units basis, no beneficial activity was demonstrable for IFN-γ44a against acute herpetic keratitis. This finding is in agreement with the earlier mentioned property of restricted species-specificity for IFN-γ.18 However, higher concentrations of IFN-γ44a should be tested for their antiHSV activity.

The indirect mechanisms of action involved in the efficacy of IFNs in the rabbit eye may be immunologic in nature. It is noteworthy that the effects are rapid because efficacy occurs even with treatments commencing after infection. The slightly decreased antiviral activity of IFN-αCon1 treatments extending over 14 days, compared with 7 days, may reflect later stimulation of counteractive mechanisms. Although indirect mechanisms are involved, efficacy occurs with topical treatments in the eye but not with intramuscular treatments,7 indicating that the indirect mechanisms are only elicited locally.

Key words: recombinant human interferon, interferon analogs, HSV-1 therapy, acute keratitis, latent HSV infection

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References


