Effect of Natural Human Interferon-Beta on the Replication of Human Cytomegalovirus

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The antiviral effect of natural human interferon-beta (HuIFN-β) against human cytomegalovirus (CMV) was evaluated in human embryonic lung fibroblasts (HEL). Natural HuIFN-β, like other HuIFNs, inhibited the replication of CMV. Pretreatment of the cells with natural HuIFN-β inhibited the appearance of immediate-early antigen (IEA) or pre-early nuclear antigen (PENA) as well as the production of infectious CMV. After a single treatment with natural HuIFN-β, intracellular 2', 5'-oligoadenylate (2-5A) synthetase activity was induced and maintained at a high level for several days. The anti-CMV effect of natural HuIFN-β correlated with the intracellular 2-5A synthetase activity.

Key words: 2-5A synthetase, human embryonic lung fibroblasts, immediate-early antigen, pre-early nuclear antigen

INTRODUCTION

Human cytomegalovirus (CMV) is a ubiquitous herpesvirus; CMV infections are usually subclinical in normal hosts. In immunocompromised hosts, however, apparent CMV infections frequently occur and result in severe diseases. Effective measures to treat or prevent the CMV infection are not available at the present time [Naraqi, 1984]. Human interferon (HuIFN) has been shown to inhibit CMV replication in vitro [Postic and Dowling, 1977; Holmes et al., 1978; Stinski et al., 1982; Rasmussen et al., 1984]. In clinical trials, little clinical efficacy was shown in the treatment of CMV infections of bone marrow transplant patients [Meyers et al., 1982; Wade et al., 1983], but prophylaxis with leukocyte-derived HuIFN-α had some effect on the prevention of serious CMV infections in renal transplant recipients [Cheeseman et al., 1979; Hirsch et al., 1983]. However, the HuIFN employed was impure and the amount too small. The antiviral effect of HuIFN on CMV infection needs to be reassessed with sufficient doses of purified HuIFNs of different types. The development of the modern technology has provided a large amount of highly purified HuIFNs. In Japan, natural HuIFN-β has been licensed for treatment of chronic active hepatitis B, in addition to malignant melanoma of the skin and glioblastoma; it is
being used in clinical trials for prevention of CMV infections in patients with renal transplantation [Takahashi et al., 1987]. In the present study, we examined the inhibitory effect of natural HuIFN-β on the replication of CMV compared with natural HuIFN-α and recombinant HuIFN-γ.

**MATERIALS AND METHODS**

**Cell and Medium**

Human embryonic lung fibroblasts (HEL) were grown in Eagle’s minimum essential medium (MEM) supplemented with kanamycin (60 μg/ml), 10% fetal bovine serum (Flow Laboratories, Inc., Rockville, MD), and 0.12% NaHCO₃. Maintenance medium for the cells contained 5%, instead of 10%, fetal bovine serum.

**Virus and Titration**

The AD169 strain of CMV was used throughout the experiments. The virus stock was prepared as follows. When more than 90% of cells in Roux bottles showed a cytopathic effect (CPE), the cells were harvested, sonicated for 15 sec at 100 watts on Sonifier Cell Disruptor Model W185 (Heat Systems-Ultrasonics, Inc., Plainview, NY), and centrifuged at 2,500 rpm for 15 min to free cell debris. The supernatant fluid was dispensed into screw-capped vials and kept at −70°C until use.

The infectivity of the virus was plaque-titrated as below. Confluent monolayers of HEL grown in wells of a 24-well plate (Corning Glass Works, Corning, NY) were inoculated in duplicate with 0.1 ml of a serially tenfold dilution of the virus stock. After 1 hr adsorption, the monolayers were overlaid with 2.25% methyl cellulose containing Eagle’s MEM, kanamycin (60 μg/ml), 5% fetal bovine serum, and 0.24% NaHCO₃ and incubated under atmosphere of 5% CO₂ at 37°C. After 14 days, the overlay was removed, and the cells were stained with 0.03% methylene blue. The number of plaques was counted with an inverted microscope.

**HuIFNs and HuIFN Treatment**

Natural HuIFN-β (Feron, Toray Industries, Inc., Tokyo) was induced in human foreskin fibroblasts by treatment with poly(I)·poly(C) in a combination with cycloheximide and actinomycin D and purified by affinity chromatography. Leukocyte-derived HuIFN-α (Nippon Kayaku Co., Ltd., Tokyo) and recombinant HuIFN-γ (Shionogi Seiyaku Co., Ltd., Osaka) were also used for comparison. The activity of each HuIFN was assayed by 50% cytopathic effect reduction method using human FL cells and vesicular stomatitis virus (VSV) [Kobayashi et al., 1982]. The activities of both HuIFN-α and HuIFN-β were expressed in international unit (IU) based on the international standard HuIFN-α and HuIFN-β (NIH, Bethesda, MD) and were 1.0 × 10⁷ IU/mg protein, respectively. The titer of recombinant HuIFN-γ was 1.0 × 10⁷ U/mg protein.

Monolayers of HEL in culture tubes received maintenance medium containing an appropriate amount of HuIFN and were incubated for 24 hr. After aspirating the medium, the virus was inoculated into the cells at a multiplicity of infection (M.O.I.) of 0.2 and adsorbed for 1 hr at 37°C. Unadsorbed viruses were aspirated off, and each culture was fed with 1 ml of maintenance medium containing the HuIFN. Untreated control cultures were inoculated with virus and treated with maintenance medium alone. Cultures were incubated at 37°C and harvested at various intervals by
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scraping the cells into medium. The pooled cell suspension was sonicated and centrifuged as described above. The supernatant fluid was stored at −70°C until plaque titration.

**Detection of CMV-Related Antigens**

Cells grown on coverslips were inoculated with CMV at an M.O.I. of approximately 2.0. For examination of cells positive for immediate-early antigen (IEA) [Michelson-Fiske et al., 1977] or pre-early nuclear antigen (PENA) [Ohtsuka et al., 1979], the infected cells were incubated in the presence or absence of various concentrations of natural HuIFN-β at 37°C for 2 hr. The cells were washed three times in phosphate-buffered saline (PBS) and air-dried. The cells were fixed in cold acetone for 5 min and kept dry at 4°C until staining. PENA was examined by anti-complement immunoperoxidase method (ACIP). The infected cells were reacted with heat-inactivated (56°C, 30 min) anti-PENA antibody-positive human serum at a 1:20 dilution.

After incubation at room temperature for 30 min in a moist chamber, the cells were washed three times (5 min each) in PBS. The cells were incubated for 30 min with human complement (anti-CMV antibody-negative serum) at a 1:20 dilution, rinsed three times in PBS, and then incubated for 30 min with anti-human complement C3 goat IgG fraction conjugated with peroxidase (Cappel Laboratories, Cochranville, PA) at a 1:50 dilution. After washing three times with PBS and dipping in 0.5 M Tris-HCl buffer (pH 7.5) for 5 min, the cells were reacted with a freshly prepared substrate solution consisting of 3,3′-diaminobenzidine tetrahydrochloride (0.05%) and hydrogen peroxide (0.01%) in Tris-HCl buffer. After washing twice with distilled water, the coverslips were dehydrated through an alcohol series to xylene, mounted with Diatex (AB Wilh Becker Industrial Paint, Marsta, Sweden), and examined under a light microscope.

For examination of early antigen (EA)-positive cells, the infected cells were incubated at 37°C for 48 hr in maintenance medium containing 50 μg/ml of cytosine arabinoside in a combination of various concentrations of natural HuIFN-β or none. To examine late antigen (LA)-positive cells, the infected cells were incubated for 72 hr at 37°C in maintenance medium, with or without various concentrations of natural HuIFN-β. EA and LA were examined by indirect immunoperoxidase method (IIP), which was performed using anti-EA and anti-LA antibody-positive human serum at a 1:20 dilution, respectively, and anti-human IgG goat F(ab')2 fragment conjugated with peroxidase (Cappel Laboratories, Cochranville, PA) at a 1:50 dilution. Production of PENA or EA was considered positive when the nuclei of infected cells were stained brown. LA was considered positive when the intranuclear inclusion body was specifically stained.

**Assay of 2′, 5′-Oligoadenylate (2-5A) Synthetase Activity**

**Cell extracts.** Cell extracts were prepared from untreated or natural HuIFN-β-treated cells as described by Merlin et al. [1981]. Briefly, after removing the medium, cells were detached by trypsin-EDTA, washed twice in ice-cold PBS, and lysed at 0°C for 15 min with 0.1 ml of lysis buffer (0.5% Nonidet-P40; 20 mM HEPES, pH 7.5; 5 mM MgCl2; 120 mM KCl; 1 mM DTT, and 10% glycerol). The lysate was centrifuged at 12,000 rpm for 6 min, and the supernatant fluid was stored at −70°C until assay.
Radioimmunoassay. Levels of 2-5A synthetase activity in the cell extracts were determined by competitive radioimmunoassay as described by Sawai et al. [1984, 1985]. The enzyme activity was assayed using an assay kit (Eiken Immunological Laboratories, Tokyo), which was reported to be useful as a simple diagnostic tool for virus infections [Sugino et al., 1986]. The kit consists of nonlabeled ATP as a substrate; poly(I)·poly(C)-agarose gel, which adsorbs and activates 2-5A synthetase; 125I-labeled 2-5A; and anti-2-5A serum to precipitate both 2-5A products used for an assay. The 2-5A synthetase activity was expressed as the yield of 2-5A for the whole cell extract of each tube culture.

Statistical Analysis

Virus yield inhibition at each time point was statistically analyzed by the Wilcoxon-Mann-Whitney test.

RESULTS

Effect of Different HuIFNs on CMV Replication

The antiviral effect of different types of natural and recombinant HuIFNs on CMV was examined first by inhibition of the virus yield. Cells were treated with various concentrations of natural HuIFN-α, natural HuIFN-β, or recombinant HuIFN-γ for 24 hr before inoculation of CMV at an M.O.I. of 0.2. Each tube culture was then maintained with HuIFN-containing medium, which was replenished every other day. HuIFNs had no cytotoxic effect on HEL, even at a concentration of 3.0 × 10^4 IU/ml (data not shown). As shown in Figure 1, each HuIFN significantly inhibited CMV replication for 3-5 days postinfection (P < .05). The higher concentrations of HuIFNs produced lower virus yields in infected cells treated with HuIFNs (P < .05). Compared with untreated control cultures, CMV titers were approximately 2-3 logs

Fig. 1. Inhibition of cytomegalovirus (CMV) replication by three types of human interferons (HuIFNs). Monolayers of human embryonic lung fibroblasts (HEL) in tubes were treated for 24 hr with various concentrations of natural HuIFN-α, natural HuIFN-β, or recombinant HuIFN-γ, or they were mock-treated; they were then infected with CMV at a multiplicity of infection (M.O.I.) of 0.2. Infected cells were harvested at the indicated day points for plaque-titration of infectious viruses.
lower in cells treated with each HuIFN for 4–5 days postinfection. Under these conditions, the three HuIFNs showed little difference in antiviral activity against CMV. Thus the replication of CMV was sensitive to HuIFNs.

Effect of Natural HuIFN-β on the Appearance of CMV-Related-Antigens and Cytomegalia

To determine the HuIFN-sensitive stage in the virus replication, natural HuIFN-β was examined for blocking the appearance of CMV-related-antigens and/or cytomegalia. As shown in Table I, production of CMV-related-antigens was strongly inhibited in cells treated with $3.0 \times 10^4$ IU/ml of natural HuIFN-β. The rates of cells positive for PENA, EA, or LA decreased with increase in dose of natural HuIFN-β applied to the cells. The appearance of PENA was more sensitive to natural HuIFN-β than that of EA or LA. Thus natural HuIFN-β seems to inhibit CMV at a very early stage of the viral replication cycle.

Comparison of Antiviral Effect of Natural HuIFN-β Treated Before and/or After CMV Infection

To examine the relationship between timing of natural HuIFN-β treatment and inhibition, cultures were pretreated or posttreated or both. The results presented in Figure 2 showed that the antiviral activity was approximately ten times greater in natural HuIFN-β-pretreated cultures than in posttreated ones ($P < .05$). The combined treatment was most effective in protecting the cells from CMV infection. Although less effective than other treatments, even posttreatment of the cells with natural HuIFN-β did exert antiviral activity against the replication of CMV ($P < .05$). Similar results were obtained for the effect of natural HuIFN-β on the appearance of CMV-related-antigens and cytomegalia (Table II). Again, posttreatment of cells with natural HuIFN-β inhibited the production of CMV-related-antigens and cytomegalia.

Relationship Between Virus Inhibition and 2-5A Synthetase Activity in Natural HuIFN-β-Pretreated HEL

We were interested in determining whether the 2-5A system was mobilized in HEL by natural HuIFN-β-treatment.

TABLE I. Effect of Natural HuIFN-β on the Appearance of CMV-Related-Antigens and Cytomegalia

<table>
<thead>
<tr>
<th>Natural HuIFN-βa (IU/ml)</th>
<th>Pre-early nuclear antigen (PENA)b</th>
<th>Early antigen (EA)b</th>
<th>Late antigen (LA) and/or cytomegaliab</th>
</tr>
</thead>
<tbody>
<tr>
<td>---</td>
<td>72.6</td>
<td>77.0</td>
<td>100</td>
</tr>
<tr>
<td>$3 \times 10^1$</td>
<td>41.7</td>
<td>35.6</td>
<td>91.8</td>
</tr>
<tr>
<td>$3 \times 10^2$</td>
<td>21.8</td>
<td>15.7</td>
<td>69.1</td>
</tr>
<tr>
<td>$3 \times 10^3$</td>
<td>2.7</td>
<td>2.9</td>
<td>37.3</td>
</tr>
<tr>
<td>$3 \times 10^4$</td>
<td>1.5</td>
<td>3.9</td>
<td>7.4</td>
</tr>
</tbody>
</table>

aMonolayers of human embryonic lung fibroblasts (HEL) on coverslips were treated with various doses of natural human interferon-beta (HuIFN-β) prior to infection with cytomegalovirus (CMV) at multiplicity of infection (M.O.I.) of approximately 2.

bPercent positive cells were determined per approximately 500 cells in different various microscopic fields.
Fig. 2. Comparison of antiviral effect of human interferon-beta (HuIFN-β) treated before and/or after cytomegalovirus (CMV) infection. Monolayers of human embryonic lung fibroblasts (HEL) in tubes were treated with $3 \times 10^4$ IU/ml of natural HuIFN-β before and/or after infection with CMV at a multiplicity of infection (M.O.I.) of 0.2: no treatment (○); posttreatment (●); pretreatment (■); pre- and posttreatment (▲). Infectious viruses were plaque-titrated on the indicated days.

After pretreatment with $3.0 \times 10^4$ IU/ml of natural HuIFN-β for 24 hr, the intracellular 2-5A synthetase activity became elevated from day 0, and the enzyme activity was maintained at high level for up to 5 days (Fig. 3A). Thereafter 2-5A synthetase activity started to decline gradually (Fig. 3B).

The enzyme levels in natural HuIFN-β-treated cells on day 14, 21, and 28 were approximately $10^2$ fmol/tube 2-5A. In contrast, the 2-5A synthetase activity was undetectable in extracts from untreated control cells.

The relationship between 2-5A synthetase activity and antiviral activity was examined. The natural HuIFN-β-pretreated cells were divided into two groups. On the designated days, one group of cells were harvested for assay of the intracellular 2-5A synthetase activity. On the same day, the other group of cells were inoculated with CMV at an M.O.I. of 0.2 and incubated in the absence of natural HuIFN-β for an additional 4 days, after which the virus yields were plaque-titrated. The virus titers thus determined were plotted against the day of infection (Figs. 3A, 3B).
TABLE II. Effect of Time of Natural HuIFN-β Treatment on the Appearance of CMV-Related Antigens and Cytomegalia

<table>
<thead>
<tr>
<th>Time of natural HuIFN-β treatmenta</th>
<th>Pre-early nuclear antigen (PENA)b</th>
<th>Early antigen (EA)b</th>
<th>Late antigen (LA) and/or cytomegaliab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre</td>
<td>Post</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−</td>
<td>−</td>
<td>9.0</td>
<td>51.6</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>1.7</td>
<td>9.6</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>0.6</td>
<td>0.7</td>
</tr>
</tbody>
</table>

aMonolayers of human embryonic lung fibroblasts (HEL) on coverslips were treated with $3 \times 10^4$ IU/ml of natural human interferon-beta (HuIFN-β) or untreated before and/or after inoculation of cytomegalovirus (CMV) at a multiplicity of infection (M.O.I.) of approximately 2.
bPercent positive cells per approximately 500 cells.

In natural HuIFN-β-pretreated cells, the virus yields remained less than 1% of the untreated cells for the first 5 days, when the 2-5A synthetase activity was constantly detected (Fig. 3A). However, as the 2-5A synthetase activity declined after 7 days, the natural HuIFN-β-pretreated cells allowed CMV to replicate up to the level of the untreated control cells (Fig. 3B). Apparently, there was a good correlation between the 2-5A synthetase activity and the degree of inhibition of CMV growth. The coefficient of correlation was 0.880 ($P < .001$; analyzed by the Student t-test).

Relationship Between CMV Replication and 2-5A Synthetase Activity in CMV-Infected HEL

Endogenous IFN would be the most likely source of IFN early in the virus infection. In the one-step growth cycle of CMV, CMV replication and 2-5A synthetase activity were measured in natural HuIFN-β-pretreated and untreated cells (Fig. 4). The pretreated and untreated cells were equally infected with CMV at an M.O.I. of 0.2; they were monitored daily for 2-5A synthetase activity and infectious virus. As seen in Figure 4, CMV infection alone induced enzyme activity to a small extent; the activity was otherwise undetectable (Fig. 3A). However, the level of activity was about one-thousandth of that detected in the cells pretreated with natural HuIFN-β and infected by CMV. On the other hand, the pretreatment by natural HuIFN-β delayed the replication of CMV and reduced the amount of infectious progeny CMV over a period of 5 days (Fig. 4).

DISCUSSION

It is well known that there are differences between the inhibitory effects of different species of IFNs. Rasmussen et al. [1984] compared the anti-CMV effect of several kinds of recombinant and native HuIFN-α and -β and showed that recombinant HuIFN-β inhibited CMV more than native HuIFN-β or several kinds of recombinant HuIFN-α. There was little difference in anti-CMV effect among natural HuIFN-α, natural HuIFN-β and recombinant HuIFN-γ, although recombinant HuIFN-β was not evaluated in our experiments.

The anti-CMV effect of natural HuIFN-β is expressed as an inhibition of a very early step in the replication cycle of CMV. The possible step of the inhibition may be
Fig. 3. Relationship between the intracellular 2-5A synthetase activity and inhibition of cytomegolavirus (CMV) replication. The cell cultures were challenged with CMV (M.O.I. = 0.2) on A: 0, 1, 2, 3, 4, and 5 days and on B: 0, 1, 7, 14, 21, and 28 days after the removal of natural human interferon-beta (HuIFN-β). All the cultures thus infected were harvested for the assay of infectious viruses after incubation for an additional 4 days. The virus titers were plotted against the time of virus inoculation. Intracellular 2-5A synthetase activity was determined on the day of virus inoculation. M.O.I., multiplicity of infection.

either viral penetration as in the case of VSV [Whitaker-Dowling et al., 1983] or the synthesis of PEN. Although it remains to be determined which is the sensitive step, Stinski et al. [1982] clearly showed that HuIFN-α/β inhibited translation of immediate early CMV-specific mRNA. The anti-CMV effect of natural HuIFN-β was prominent when the cells were pretreated, but even posttreatment of cells after infection also interfered with the synthesis of PEN. Therefore, short-time treatment with natural HuIFN-β made cells resistant to CMV infection.

In contrast with recombinant HuIFN-γ [Yamamoto et al., 1987], the anti-CMV state of natural HuIFN-β-treated cells correlated with the level of intracellular 2-5A synthetase; 2-5A synthetase, in particular, plays an important role in the antiviral effect of IFN [Baglioni, 1979; Lengyel, 1982]. Koliais et al. [1982] reported that sensitivity to IFN correlated with the level of 2-5A synthetase after induction with
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Fig. 4. Effect of natural human interferon-beta (HuIFN-β) pretreatment on 2-5A synthetase activity and virus yields. Monolayers of human embryonic lung fibroblasts (HEL) in tubes were treated with $3 \times 10^4$ IU/ml of natural HuIFN-β for 24 hr or untreated prior to virus inoculation at a multiplicity of infection (M.O.I.) of 0.2. The infected cultures were monitored daily up to 5 days for intracellular 2-5A synthetase activity and for infectious viruses.

IFN in lymphoid cells. These data support the conclusion obtained in our experiments. Viral sensitivity to exogenous IFN, however, is variable, and the response of cells to IFN differs, as has been suggested by Sen and Herz [1983]. For example, in mouse embryonic carcinoma cells, IFN treatment induced 2-5A synthetase activity, but, for some viruses, the antiviral state is not achieved [Wood and Hovanessian, 1979; Nilsen et al., 1980].

Verhaegen et al. [1980] detected the presence of high levels of 2-5A synthetase in a human cell line (HEC-1), with or without IFN treatment, but the 2-5A synthetase could not block the replication of VSV and sindbis virus. On the other hand, although in MRC5 cells (human diploid fibroblasts derived from a female embryonic lung) treated or untreated with IFN, 2-5A synthetase is not detectable, the growth of VSV was inhibited [Meurs et al., 1981]. There is a difference in the ability to acquire an antiviral state and 2-5A synthetase activity between lymphoid cell lines [Adams et al., 1975]. The intracellular events leading to the development of the antiviral
response may vary with host cells. Mechanisms excepting the 2–5A may exist in the antiviral effect of IFN, but it remains poorly understood.

Natural HuIFN-β has been licensed in Japan to treat malignant melanoma, glioblastoma, and chronic active hepatitis B. As a trial for expanding its clinical application, natural HuIFN-β also was used to treat CMV pneumonitis in patients with renal transplantation [Takahashi et al., 1987]. In that trial, all eight patients with CMV pneumonitis were cured, though there were some reversible side-effects such as fever, slight hepatic dysfunction, and proteinuria. In these patients, the serum level of natural HuIFN-β reached 30 IU/ml and then declined to less than 6 IU/ml within a few hours. Natural HuIFN-β was actually effective against CMV in vitro, even at a concentration of 30 IU/ml, and once treated with natural HuIFN-β, the cells were resistant to CMV for 5 days. Therefore, continuous supply of exogenous HuIFN-β or maintenance of a certain level of HuIFN-β may not be necessary to control CMV infection in vivo and in vitro. The results of our in vitro study, together with that of in vivo cited above, strongly suggest the clinical effectiveness of natural HuIFN-β against CMV infections.

ACKNOWLEDGMENTS

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