Stimulation of Fibroblast Interferon Production by a 22K Protein from Human Leukocytes

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SUMMARY

We have studied the appearance of human interferon-β (HuIFN-β) as well as its mRNA in cells treated with a protein, 22K factor, isolated from the culture supernatant of mitogen-stimulated human peripheral blood leukocytes. By itself 22K was found to be unable to induce production of significant amounts of HuIFN-β protein. However, when aided by treatment with cycloheximide or cycloheximide and actinomycin D (superinduction), 22K caused increases in production ranging from 3- to 20-fold, depending on the cells (diploid or MG-63 osteosarcoma) and the induction schedule. Cells treated with 22K alone produced small amounts of HuIFN-β mRNA, which was only detectable with a highly sensitive method. In combination with cycloheximide, 22K induced levels of mRNA detectable with less sensitive methods as well. These experiments provide further support for the concept that the antiviral activity of 22K is mediated by its ability to stimulate transcription of the HuIFN-β gene in cells.

INTRODUCTION

We have previously reported that human peripheral blood leukocytes, stimulated with concanavalin A (Con A) produce an antivirally active protein with a molecular weight, on gel filtration, of 22000 (Van Damme et al., 1981). The protein was provisionally called ‘22K factor’. In contrast to interferon-γ (HuIFN-γ), which is also produced by these leukocytes but which, on gel filtration, behaves as a protein of 45000 mol. wt., the 22K factor was stable in acid and SDS-containing buffers. Its antiviral activity could not be neutralized with antibody against HuIFN-α or HuIFN-γ, but was completely neutralizable with specific antibody against HuIFN-β. Nevertheless, the 22K factor could be distinguished from HuIFN-β by its cell specificity and its different behaviour on zinc chelate and Con A–Sepharose chromatography.

Some observations indicated that the 22K factor was not a variant of HuIFN-β, but rather an interferon-inducing protein (Van Damme et al., 1983): failure to bind to a column of antibody against HuIFN-β and lack of neutralization of HuIFN-β by antibody against 22K. We here provide evidence which further supports this statement.

METHODS

Production and purification of the 22K factor. Human peripheral blood leukocytes obtained from pooled blood donations were stimulated with concanavalin A (10 μg/ml) for 48 h. Crude supernatant was concentrated and partially purified by adsorption to silicic acid (Van Damme et al., 1981). Impurities were further removed by adsorption to DEAE-Sephacel at pH 8.0: the unadsorbed material was then fractionated by gel filtration on a Sephacryl S-200 column, yielding a specific activity of >106 antiviral units (AVU) per mg protein.

Cell cultures and induction of HuIFN-β. The human osteosarcoma cell line (MG-63) and the human diploid fibroblast cell strain (E6SM) were cultured in modified Eagle’s minimum essential medium (Gibco), supplemented with 10% heat-inactivated foetal bovine serum. The cells were grown as monolayers in 175 cm² plastic flasks (Falcon no. 3045). Confluent cell cultures, obtained after 1 week incubation at 37 °C, were used for the induction experiments.
Parallel groups of cell cultures were induced with 30 ml of medium containing either polyriboinosinic:ribocytidylic acid (poly-rI : rC) (50 µg/ml; P-L Biochemicals) or purified 22K protein (>10^2 AVU/ml) in the presence or absence of cycloheximide (10 µg/ml; Serva, Heidelberg, F.R.G.). For the extraction of HulIFN-β mRNA, cells were treated as described below after 4 h incubation at 37 °C. For the production of HulIFN-β protein, after 4 h incubation cell cultures were further incubated (superinduction) in the presence of actinomycin D (1 µg/ml, Serva) for 2 h. At the end of the superinduction period cell cultures were washed three times with phosphate-buffered saline (PBS) and re-incubated for 24 h with 30 ml of medium containing 2% foetal bovine serum.

**Extraction and measurement of RNA.** The mRNA extraction procedure was modified from that previously described (Opdenakker et al., 1982). Confluent cultures of cells, growing in monolayers, were induced as described and suspended in PBS without calcium chloride or magnesium chloride, supplemented with 0-04% EDTA. Groups of treated and untreated cells were pelleted and lysed in a hypotonic buffer (10 mM-NaCl, 3 mM-magnesium acetate, 20 mM-Tris-HCl pH 7-4), containing ribonucleosyl-vanadyl complexes (Bethesda Research Laboratories) to inhibit RNase. Usually 10 ml of buffer was used per 10^9 cells. After thorough mixing, nuclei were removed by centrifugation and the cytoplasmic supernatant was extracted with phenol and with a mixture of phenol, chloroform and isoamyl alcohol as described by Berger & Birkenmeier (1979). Total RNA was precipitated by addition of NaCl to 0-2% and 2 vol. absolute ethanol overnight at −20 °C. In some of the experiments it was subsequently fractionated by affinity chromatography on oligo(dT)-cellulose (Aviv & Leder, 1972) prior to the Northern blot assays and translation experiments. RNA was measured spectrophotometrically (A_{260}/A_{320}). Since the vanadyl complexes present in the total RNA preparations, but not in purified RNA samples, interfered with absorbance at 260 nm, only relative comparisons between different groups of total RNA preparations were made.

**Translation in oocytes and assay of HulIFN-β.** RNA from differently treated cell cultures was extracted and purified as described by Opdenakker et al. (1982). Poly(A)^+ RNA was injected and translated in *Xenopus laevis* oocytes for 48 h as described. Aliquots of 50 ml poly(A)^+ RNA at 1 mg/ml were injected per oocyte. Groups of 10 oocytes were incubated in 100 µl Barth's medium (Gurdon et al., 1971). Supernatant fluids from oocytes or cell cultures were tested for HulIFN-β activity with a viral cytopathogenicity reduction method on EoSM or HEP-2 cells using vesicular stomatitis virus as challenge virus (Van Damme et al., 1981). Antiviral activities were seroneutralized by incubating a constant amount of specific antiserum against HulIFN-β (Van Damme et al., 1981) with each dilution in the interferon assay for 2 h at 37 °C.

**Measurement of HulIFN-β mRNA.** The RNA preparations were also tested by nitrocellulose dot blot assay (Kafatos et al., 1979). Equal amounts of the RNA preparations were denatured (heating for 5 min at 70 °C followed by quick cooling at 0 °C) and suspended in 15 × SSC (1 × SSC is 0-15 M-NaCl, 0-015 M-sodium citrate pH 7-4). Tenfold dilution series were made in 15 × SSC in microtitre plates, leaving 100 µl per dilution. After wetting the nitrocellulose membrane (HAHY 00010 from Millipore or BA85 from Schleicher & Schüll) in double-distilled water and presoaking in 20 × SSC, the RNA dilution series were applied to the filter, using a manifold dot blot apparatus (Schleicher & Schüll). The filters were baked for 3 h at 85 °C. The HulIFN-β RNA probe was prepared by transcribing 1 µg of EcoRI-linearized pSP6-1F^+ plasmid DNA (a kind gift of Drs K. Zinn and T. Maniatis) with the SP6 RNA polymerase, as described by Zinn et al. (1983), except that unincorporated nucleotides were removed by Sephadex G-100 chromatography (Green et al., 1983). Filter prehybridization, hybridization with the labelled RNA probe and washings were done according to published procedures (Zinn et al., 1983). This protocol allows detection of 1 pg specific DNA per dot after a 24 h exposure.

The presence of HulIFN-β mRNA was also tested by Northern blot analysis (Alwine et al., 1977). Samples of polyadenylated RNA were subjected to electrophoresis in 1-1% agarose as described by Thomas (1980). Because only small amounts of RNA (<2 µg) were available, minigels (5 × 7 × 0-3 cm) were used. After transfer to nitrocellulose the filters were hybridized with a highly radiolabelled RNA probe as described above. Ribosomal ^3^P-labelled RNA markers were prepared and used as described earlier (Content et al., 1983).

**RESULTS**

**Induction of HulIFN-β in human fibroblastoid cells by the 22K factor**

Groups of confluent diploid fibroblast (EoSM) and human osteosarcoma (MG-63) cell cultures were incubated for 2 h with either poly-rI : rC (50 µg/ml) or 22K (>10^2 AVU/ml), or were left untreated. Parallel cell cultures were induced with poly-rI : rC or 22K in the presence of the metabolic inhibitor cycloheximide (10 µg/ml) for 4 h. Furthermore, as part of the superinduction schedule (Billiau et al., 1973), other cell cultures induced with poly-rI : rC or 22K in combination with cycloheximide received a further treatment with actinomycin D (1 µg/ml) for 2 h. At the end of their different induction schedules, all cell cultures, including the non-induced control groups, were left untreated. Parallel groups of cell cultures were induced with 30 ml of medium containing either polyriboinosinic:ribocytidylic acid (poly-rI : rC) (50 µg/ml; P-L Biochemicals) or purified 22K protein (>10^2 AVU/ml) in the presence or absence of cycloheximide (10 µg/ml; Serva, Heidelberg, F.R.G.). For the extraction of HulIFN-β mRNA, cells were treated as described below after 4 h incubation at 37 °C. For the production of HulIFN-β protein, after 4 h incubation cell cultures were further incubated (superinduction) in the presence of actinomycin D (1 µg/ml, Serva) for 2 h. At the end of the superinduction period cell cultures were washed three times with phosphate-buffered saline (PBS) and re-incubated for 24 h with 30 ml of medium containing 2% foetal bovine serum.
Table 1. Interferon production by cells pretreated with 22K in the presence or absence of cycloheximide and/or actinomycin D

<table>
<thead>
<tr>
<th>Adjuvant treatment</th>
<th>Cycloheximide† + actinomycin D</th>
</tr>
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<tbody>
<tr>
<td>Inducer</td>
<td>None*</td>
</tr>
<tr>
<td>E6SM diploid cells</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.8§</td>
</tr>
<tr>
<td>Poly-rI : rC (50 µg/ml)</td>
<td>2.2</td>
</tr>
<tr>
<td>22K (≥ 10^2 AVU/ml)</td>
<td>1.1</td>
</tr>
<tr>
<td>MG-63 cells</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1.1</td>
</tr>
<tr>
<td>Poly-rI : rC (50 µg/ml)</td>
<td>3.0</td>
</tr>
<tr>
<td>22K (≥ 10^2 AVU/ml)</td>
<td>1.7</td>
</tr>
</tbody>
</table>

* Cell cultures were induced with poly-rI : rC or 22K for 2 h, washed three times with PBS and incubated overnight with growth medium.
† Parallel cell cultures were induced in the presence of cycloheximide (10 µg/ml) for 4 h and further treated as in (*).
‡ Cell cultures were induced for 4 h, as in (†) then incubated with actinomycin D (1 µg/ml) for 2 h, and further processed as in (*).
§ Antiviral activity (log_{10} U/ml) as determined on diploid (left) and HEp-2 cells (right); values are means of two experiments.

The results of these experiments are summarized in Table 1. Without any treatment, interferon production was low in MG-63 and in E6SM cells. The adjuvant treatment with cycloheximide and actinomycin D caused enhancement of this spontaneous production. The results with poly-rI : rC as an inducer were as expected: reasonably good production with poly-rI : rC only, enhancement by addition of cycloheximide or by superinduction (i.e. addition of both cycloheximide and actinomycin D). In diploid cells 22K caused an ambiguous response: without adjuvant(s) no effect was seen; in the presence of cycloheximide a threefold increase above background of the cycloheximide control group was detectable. With superinduction, 22K did not seem to enhance interferon production, as compared to the control group treated with cycloheximide and actinomycin D. In MG-63 cells, known for their good inducibility (Billiau et al., 1977), 22K caused a 20-fold increase of interferon production in the presence of cycloheximide or with the superinduction method (as compared to the appropriate control groups cycloheximide alone or cycloheximide and actinomycin D alone).

In order to characterize the antiviral activity produced in fibroblastic cells after treatment with 22K, neutralization tests were performed using specific antisera against HuIFN-β and 22K. Table 2 shows that the antiviral activity obtained in MG-63 cells by the superinduction schedules for HuIFN-β (using poly-rI : rC, cycloheximide and actinomycin D) was completely

Table 2. Identification of 22K-induced antiviral activity

<table>
<thead>
<tr>
<th>Antiviral activity (log_{10} U/ml) on Diploid cells</th>
<th>HEp-2 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Induction*</td>
<td>No</td>
</tr>
<tr>
<td>None</td>
<td>2.5</td>
</tr>
<tr>
<td>Poly-rI : rC</td>
<td>4.3</td>
</tr>
<tr>
<td>22K</td>
<td>3.5</td>
</tr>
<tr>
<td>HuIFN-β standard</td>
<td>3.5</td>
</tr>
<tr>
<td>22K standard</td>
<td>3.8</td>
</tr>
</tbody>
</table>

* MG-63 cells were tested for HuIFN-β production using the superinduction method with poly-rI : rC (50 µg/ml) or 22K (≥ 10^2 AVU/ml), cycloheximide (10 µg/ml) and actinomycin D (1 µg/ml) as described in Methods.
† Constant concentration of antibody added to each dilution of the interferon assay for 2 h at 37°C.
Table 3. *HuIFN-β* activity in MG-63 culture fluid and detection of *HuIFN-β* mRNA in parallel cultures by translation in oocytes

<table>
<thead>
<tr>
<th>Treatment of MG-63 cells*</th>
<th><em>HuIFN-β</em> (log_{10} U/ml) in culture fluid</th>
<th>Poly (A)* RNA content (ng/10^6 cells)</th>
<th>HuIFN-β antiviral activity (log_{10} U/ml) in oocyte fluids†</th>
<th>Residual (with antibody)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.3</td>
<td>800</td>
<td>&lt;0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Cycloheximide (10 μg/ml)</td>
<td>1.0</td>
<td>560</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Poly-rI:rC (50 μg/ml)</td>
<td>3.6</td>
<td>400</td>
<td>1.6</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>+ cycloheximide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22K (&gt;10^2 AVU/ml)</td>
<td>1.3</td>
<td>560</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>22K + cycloheximide</td>
<td>2.1</td>
<td>800</td>
<td>1.4</td>
<td>0.5</td>
</tr>
<tr>
<td><em>HuIFN-β</em> standard</td>
<td>4.2</td>
<td>360</td>
<td></td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>

* For extraction of mRNA, MG-63 cells were induced for 4 h; for production of interferon, cells were further treated with actinomycin D for 2 h, washed, refed and incubated for 16 h.
† Groups of 10 *X. laevis* oocytes were injected with 50 nl aliquots of preparations containing 1 μg/μl poly(A)* RNA from MG-63 cells. After 48 h incubation in 100 μl of Barth’s medium, the fluids were supplemented with 10% foetal calf serum and titrated for *HuIFN-β* activity on E6SM cells.
‡ Seroneutralization with anti-*HuIFN-β* serum, for 2 h at 37°C.

neutralized by an antiserum against *HuIFN-β*. Similarly, the 22K-induced activity was neutralized by the same antiserum. To exclude the possibility that the antiviral activity seen in the interferon assay was due to residual 22K from the induction fluid itself, the production fluids were titrated in the presence of a specific antiserum against 22K (Table 2). The characterization tests were done on diploid cells and also on HEp-2 cells, the latter being insensitive to 22K by itself. The results show that 22K-induced antiviral activity was active in HEp-2 cells. Furthermore, the antiviral activity was not neutralizable by anti-22K serum on either diploid fibroblasts or HEp-2 cells.

**Induction of *HuIFN-β* mRNA by 22K and cycloheximide:** a transcriptional effect

The following experiments were done to see whether 22K causes an increase in the cellular level of *HuIFN-β* mRNA. Groups of 10 confluent monolayers of MG-63 cells were treated for 4 h with 22K (>10^2 AVU/ml), or poly-rI:rC (50 μg/ml) either alone or in combination with 10 μg/ml of cycloheximide. Control cultures were left untreated. Cell lysis and RNA extraction were done as described in Methods. Poly(A)* RNA yields were determined. Since they varied considerably between treated groups, the concentrations were adjusted before injection into oocytes. The results from this experiment are summarized in Table 3. As a positive control it was verified that poly-rI:rC in combination with cycloheximide did induce production of interferon mRNA. 22K alone did not cause a significant increase in the amount of *HuIFN-β* produced; mRNA for *HuIFN-β* activity also remained undetectable. The combination of 22K with cycloheximide induced both increased production and appearance of biologically active mRNA. The biological activity of these interferons was completely neutralized with a specific anti-*HuIFN-β* serum.

Because neither cycloheximide nor 22K alone could induce *HuIFN-β* mRNA, it was not clear from this experiment which of the two was the inducer of mRNA in the combined treatment. Also, since this technique only indirectly measured the mRNA for *HuIFN-β*, specific mRNA was directly measured by dot blot hybridization. Confluent cultures of MG-63 cells and diploid E6SM cells were treated with 22K or with poly-rI:rC. These treatments were done both in the presence and absence of cycloheximide. After incubation for 4 h the treated cell cultures were lysed and total RNA was extracted. Equal amounts of the total cytoplasmic RNA preparations were bound to nitrocellulose filters and hybridized to a radioactively labelled *HuIFN-β* probe. Fig. 1(a) shows a nitrocellulose dot blot from MG-63 cells. Not only poly-rI:rC but also
Fig. 1. HuIFN-β mRNA dot blot hybridization of MG-63 cells and E6SM cells. Confluent cultures of MG-63 (a) and E6SM cells (b) were treated with 22K factor (≥ 10^2 AVU/ml), poly-rI:rC (50 μg/ml), cycloheximide (10 μg/ml) or HuIFN-β (200 AVU/ml). Total cytoplasmic RNA was extracted and assayed by dot blot hybridization with a radioactively labelled HuIFN-β mRNA probe. Assay mixture for each lane was as follows. (a) MG-63 cells: (1) control, not treated, (2) cycloheximide, (3) poly-rI:rC and cycloheximide, (4) 22K factor and cycloheximide, (5) HuIFN-β and cycloheximide, (6) poly-rI:rC, (7) 22K factor, (8) HuIFN-β, (b) E6SM cells: (1) control, (2) 22K factor, (3) 22K factor 1/10, (4) poly-rI:rC, (5) HuIFN-β, (6) cycloheximide, (7) 22K factor and cycloheximide, (8) 22K factor 1/10 and cycloheximide, (9) poly-rI:rC and cycloheximide, (10) HuIFN-β and cycloheximide. Quantities of RNA per dot are indicated in μg.
Fig. 2. Northern blot analysis of poly(A)+ RNA from MG-63 cells. Confluent cultures of MG-63 cells were treated with 22K (> 10^2 AVU/ml), poly-rI:rC (50 μg/ml), cycloheximide (10 μg/ml) or HuIFN-β (200 AVU/ml). Total RNA was extracted and purified by affinity chromatography on oligo(dT)-cellulose. Poly(A)+ RNA was electrophoretically separated on a 1.1% agarose gel, transferred to a nitrocellulose membrane and hybridized with a HuIFN-β RNA probe. Lanes from autoradiography (a) are as follows: (1) control, not treated, (2) cycloheximide, (3) poly-rI:rC and cycloheximide, (4) 22K factor, (5) 22K factor and cycloheximide. The following are also indicated: 28S and 18S ribosomal RNA markers; 3.5 kb and 1.8 kb RNA as described by Content et al. (1983), Raj et al. (1983) and May et al. (1983) and the major 1.1 kb HuIFN-β mRNA. An autoradiograph from an independent experiment is shown in (b) and (c). Lanes are as follows: (1) HuIFN-β, (2) HuIFN-β and cycloheximide, (3) poly-rI:rC, (4) poly-rI:rC and cycloheximide, (5) control, (6) cycloheximide, (7) 22K factor and (8) 22K factor and cycloheximide. After hybridization the filter from (b) was exposed for 3 days. Lane 4 was exposed for 2 h and all other lanes for 5 days to generate (c).
cycloheximide induced levels of HuIFN-β mRNA well above background. The combination of both gave higher levels as expected. The 22K factor by itself did not provoke synthesis of mRNA measurable by this technique. The combination of 22K with cycloheximide induced a level of HuIFN-β mRNA that was about 30-fold higher than that obtained with cycloheximide alone. A similar experiment was done with human diploid cell cultures. It was expected that the induction would be less pronounced than in MG-63 cells. The dot blot autoradiograph (Fig. 1b) shows measurable induction after treatment with 22K in combination with cycloheximide and a strong induction after induction with poly-rI : rC in the presence of cycloheximide as compared with cycloheximide treatment alone.

As a control to test the possibility that 22K might act as a primer for 'induction' by cycloheximide, cells were treated with HuIFN-β (a classical priming agent on fibroblasts: Billiau et al., 1973) alone or in combination with cycloheximide. As can be seen in Fig. 1 no induction of IFN-β mRNA was detected in either case.

Characterization of the HuIFN-β mRNA induced by the 22K factor

For more careful characterization, the poly(A)+ RNA preparations from some experiments were subjected to Northern blot analysis. The results from two experiments on MG-63 cells are shown in Fig. 2. Fig. 2(a) shows that hybridizable RNA induced with poly-rI : rC plus cycloheximide or 22K plus cycloheximide migrated at a position corresponding to 1.1 to 1.2 kb, characteristic for HuIFN-β mRNA. In both these experiments 22K by itself induced measurable amounts of IFN-β mRNA. Cycloheximide alone did not induce mRNA but, as expected, caused increases in the levels induced by 22K. Also, as was seen in the dot blot analyses, cells treated with HuIFN-β to control for 'induction' by cycloheximide did not produce IFN-β mRNA.

DISCUSSION

Our first attempts to demonstrate the production of HuIFN-β in diploid cell cultures after treatment with the 22K factor were unsuccessful (Van Damme et al., 1983). In the studies reported here we used the superinduction procedure, i.e. combined treatment of cells with the 22K factor and metabolic inhibitors (cycloheximide and actinomycin D). Both production of HuIFN-β protein by the cells and appearance of intracellular HuIFN-β mRNA were studied.

Treatment of the cells with cycloheximide alone caused release of some HuIFN-β. Addition of the 22K factor caused an additional threefold increase in diploid cells and a 20-fold increase in MG-63 cells. With the superinduction method (cycloheximide plus actinomycin D), similar results were obtained. The 22K-induced antiviral activity was always completely neutralizable with specific antibody against HuIFN-β. In contrast, with an antiserum against 22K no neutralization was observed, indicating that the antiviral activity produced was not due to remaining 22K factor used as inducer.

The appearance of IFN-β mRNA in cells treated with 22K was examined with three methods: translation to form interferon in Xenopus oocytes, dot blot hybridization and Northern blot analysis using highly sensitive (Melton et al., 1984) HuIFN-β RNA probes. Oocyte translation or dot blot hybridization did not allow detection of mRNA in cells treated with 22K alone. Northern blot analysis gave positive results in three of four independent experiments. Moreover, the combination of 22K plus cycloheximide gave positive signals in all experiments, regardless of the technique of analysis. In accordance with reports in the literature (Ringold et al., 1984; Maroteaux et al., 1983; Tan & Berthold, 1977) that cycloheximide by itself can activate the interferon-β system, we occasionally found detectable IFN-β mRNA in cells treated with cycloheximide alone. However, this occurred in only two out of eight experiments and the levels induced were much lower than those obtained with the combination of 22K plus cycloheximide. These experiments clearly show that 22K has the ability to stimulate transcription of IFN-β mRNA.

The possibility that this stimulation is due to priming is considered unlikely, since a classical primer, HuIFN-β, was unable to enhance constitutive or cycloheximide-mediated IFN-β mRNA production. We therefore favour the interpretation that 22K acts as an inducer of
HulFN-β and that this fully explains its antiviral activity. However, we do not postulate that 22K is an interferon inducer in the sense that this would be its main and physiologically most important function. From work in progress, it appears that 22K has several other biological activities which overshadow its antiviral and interferon-inducing potential.

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