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Characteristics of Augmentation of Cell-Mediated Cytotoxic Activities by Recombinant Human Interferon-Gamma (Met–Gln Form): A Comparative Study with Natural Human Interferon-Alpha and -Beta

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The effects of recombinant human interferon (IFN)-gamma (Met–Gln form), which has the same amino acid sequence as natural human IFN-gamma except for the N-terminal methionine, on various cell-mediated cytotoxicities were studied *in vitro* comparatively with those of natural human IFN-alpha and -beta. The augmentations of natural killer (NK) and antibody-dependent cell-mediated cytotoxic (ADCC) activities against tumor cells by IFN-gamma (Met–Gln form) were equivalent or somewhat inferior to those by the natural IFNs. However, the augmentation of ADCC activity against chicken red blood cells (CRBCs) by IFN-gamma (Met–Gln form) was superior to those by natural IFNs. This prominent effect of IFN-gamma (Met–Gln form) was considered to be mediated by monocytes contained in the mononuclear cell preparation used as effector cells. As expected, IFN-gamma (Met–Gln form) actually augmented antibody-dependent monocyte-mediated cytotoxicity against CRBCs more effectively than the natural IFNs. IFN-gamma (Met–Gln form) also enhanced macrophage-mediated tumor cytotoxicity, like other preparations of recombinant IFN-gamma. The NK-augmentation by IFN-gamma (Met–Gln form) was further increased by combination with indomethacin, and the effect was completely reversed by further addition of prostaglandin E_2 (PGE_2). However, these agents had no effect on the NK-augmentation by natural IFN-beta. These results suggest that IFN-gamma (Met–Gln form) activates macrophages (rather than NK and killer (K) cells) to kill tumor cells and release PGs more effectively than IFN-alpha and -beta.

Keywords——interferon; monocyte; lymphocyte; cytotoxicity; prostaglandin

Introduction

It is well known that interferons (IFNs) are divided into three distinct classes, *i. e.* IFN-alpha, -beta and -gamma, according to their molecular structures and biological properties. Among them, IFN-gamma, which is called "immune interferon," has been thought to play an important role in the immune system. However, studies of IFN have been limited in the past due to difficulty in obtaining large amounts of highly purified preparations. Since Gray and coworkers first succeeded in cloning human IFN-gamma genes,[1] the use of purified recombinant human IFN-gamma preparations has become possible for immunological studies, eliminating the effects of other lymphokines which might be present as contaminants of IFNs purified from natural sources.

Numerous studies have demonstrated the augmentation of cell-mediated cytotoxic activities including natural killer (NK) and antibody-dependent cell-mediated cytotoxic (ADCC) activities using various IFN preparations,[2] but there are only a few reports of such augmentations by highly purified recombinant human IFN-gamma.[3] Although in some of these studies IFN-alpha and -beta were comparatively examined with IFN-gamma, the results obtained are not consistent as regards relative activities because of differences of the IFN

preparations and experimental conditions used in these studies.[3] In addition, recombinant human IFN-gamma (Cys–Tyr–Cys–Gln form),[1] which has Cys–Tyr–Cys–Gln at the terminal has been used in most of the studies.[3] Rinderknecht *et al.* have recently shown that natural human IFN-gamma has Gln as its N-terminal residue, not Cys–Tyr–Cys–Gln.[4]

In the present study, we used recombinant human IFN-gamma (Met–Gln form) which has the same amino acid sequence as natural human IFN-gamma except that the N-terminal residue is Met, and examined the effects of this IFN-gamma on human NK, ADCC and antibody-dependent monocyte-mediated cytotoxic (ADMC) activities comparatively with those of natural human IFN-alpha and -beta under the same experimental conditions. We also attempted to confirm an augmenting effect of IFN-gamma (Met–Gln form) on macrophage-mediated tumor cytotoxic (MTC) activity because recent studies have demonstrated that IFN-gamma is one of the macrophage-activating factors (MAFs) in both man and mice.[5] If monocytes activated with IFN-gamma (Met–Gln form) release prostaglandins (PGs) (it is well known that activated macrophages release PGs),[6] it is likely that the PGs would suppress the cytotoxic ability of NK cells in the mononuclear cell preparation used as effector cells in NK assay and interfere with the NK-augmentation induced by IFN-gamma (Met–Gln form). Therefore, we treated these effector cells with indomethacin, an inhibitor of PG synthesis in cells, to examine the effect on the NK-augmentation induced by IFN-gamma (Met–Gln form) comparatively with that by natural human IFN-beta.

Experimental

Tumor Cells——We used three human cell lines. HMV-1, an amelanotic melanoma cell line, was received through the courtesy of Dr. M. Sekiguchi, Department of Clinical Oncology, Institute of Medical Science, University of Tokyo. A-549, a lung carcinoma cell line, was supplied by Genentech, Inc. (South San Francisco, U.S.A.). K-562, a myeloid leukemia, was supplied by Dr. S. Kobayashi of our laboratories.

Interferons——Recombinant human IFN-gamma (specific activity: 2.72×10^7 unit/mg protein, % purity (sodium dodecyl sulfate–polyacrylamide gel electrophoresis): 99.9%, lymulus amoebocyte lysate: 0.03 ng/mg protein) was supplied by Genentech, Inc. (South San Francisco, U.S.A.). Natural human IFN-beta (1.0×10^7 unit/mg protein) was prepared by the superinduction method using human diploid foreskin fibroblasts in our laboratories as previously reported.[7] Natural human IFN-alpha (3.72×10^6 unit/mg protein) was kindly provided by Dr. K. Cantell (Central Public Health Laboratory, Helsinki, Finland). The potency units of these IFNs were expressed as antiviral activity determined by the 50% cytopathic effect reduction assay using FL cells and vesicular stomatitis virus or Sindbis virus.

Preparation of Effector Cells——Blood of healthy volunteers was collected by venipuncture in heparinized syringes. The blood was diluted with an equal volume of Dulbecco's phosphate-buffered saline without Mg^{2+} and Ca^{2+} (Dulbecco PBS(−)) and layered over Lymphoprep (Nyegaad, Oslo, Norway). After centrifugation for 30 min at $400 \times g$, the interface mononuclear cells were removed with a pipette, washed three times with Dulbecco PBS(−), and then suspended in RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan) containing heat-inactivated 10% fetal calf serum (FCS, Commonwealth Serum Laboratories, Melbourne, Australia) for NK and ADCC assays. Monocytes for ADMC and MTC assays were separated from mononuclear cell suspensions by using a macrophage separating kit (MSP-P as a macrophage separating plate and MSP-E as an exfoliation agent, Japan Immunoresearch Laboratories, Takasaki, Japan). The monocytes were washed three times with Dulbecco PBS(−) and resuspended in RPMI 1640 medium containing 10% FCS. Monocyte purity in the monocyte suspension was >90% as determined by esterase and Giemsa staining.

Treatment of Mononuclear Cells or Monocytes with IFN——One milliliter of cell suspension containing 1.2×10^6 mononuclear cells and an equal volume of IFN solution or control solution were added to Falcon 15 ml plastic tubes. These tubes were incubated for 20 h at 37 °C in a humidified atmosphere of 95% air–5% CO_2. After the incubation, these cells were washed three times with RPMI 1640 medium containing 10% FCS and suspended in the medium. For ADMC and MTC assays, 100 μl of monocyte suspension containing $2—3 \times 10^4$ cells and 100 μl of IFN or control solution were added to Nunc plastic tissue culture plates (U-bottomed, 96 wells; Inter Med., Denmark). These plates were incubated under the same conditions as described above. After the incubation, the monocytes were washed three times by the aspiration technique, and 100 μl of RPMI 1640 medium containing 10% FCS was added to each well. In the experiment to examine the PGE_2 effect on NK activity, PGE_2 (synthesized in our laboratories) and indomethacin (Sigma, St. Louis, U.S.A.) were dissolved in the IFN solution at concentrations of 10^{-8} and 10^{-6} M, respectively.

Antisera——Rabbit anti-A-549 or anti-HMV-1 antiserum was prepared by the subcutaneous injection of 1×10^7 A-549 or HMV-1 cells suspended in 1 ml of Freund's complete adjuvant (Iatron Laboratories, Tokyo, Japan). The

injection was repeated four times at monthly intervals and antisera obtained were stored at −80 °C. Before use, the antisera were heated at 56 °C for 30 min.

^{51}Cr- or ^{125}I-Labelling of Target Cells——For NK assay, K-562 cells were labelled by a 1-h incubation in 500 μl of RPMI 1640 medium containing 100 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, U.S.A.) and 2% FCS at 37 °C in humidified atmosphere of 95% air–5% CO_2. After the incubation, the ^{51}Cr-labelled cells were washed three times with and suspended in RPMI 1640 medium containing 10% FCS. In NK or ADCC assay, 1×10^4 A-549 or HMV-1 cells suspended in 100 μl of Dulbecco's modified Eagle medium (Nissui Seiyaku, Tokyo, Japan) or Eagle MEM medium (Nissui Seiyaku, Tokyo, Japan) containing 10% precolostrum newborn calf serum (PNCS, Mitsubishi Chemical Industries, Tokyo, Japan) were seeded into each well of Nunc plastic tissue culture plates (U-bottomed, 96 wells) and incubated overnight under the same conditions as used for K-562 cells. After the incubation, 100 μl of RPMI 1640 medium containing 2 μCi of $Na_2{}^{51}CrO_4$ and 10% FCS were added to each well and these plates were further incubated for 3 h. The excess radioactivity was discarded by washing the cells three times, and finally, 100 μl of RPMI 1640 medium containing 10% FCS was added to each well. In the MTC assay, A-549 cells in the exponential growth phase were incubated with Dulbecco's modified Eagle medium containing 5 μCi of ^{125}I-iododeoxyuridine (Amersham International, Buckinghamshire, England) and 10% PNCS for 24 h under the same conditions as above. After the incubation, ^{125}I-labelled cells were washed with Dulbecco PBS(−), harvested by a 1-min trypsinization using 0.25% trypsin solution containing 0.02% ethylenediaminetetraacetic acid (EDTA), and suspended in RPMI 1640 medium containing 10% FCS. CRBCs used as target cells in ADCC and ADMC assay were prepared from heparin-treated fresh whole blood of female White Leghorn chickens. After being washed, 5×10^7 CRBCs were incubated for 3 h in 600 μl of RPMI 1640 medium containing 300 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, U.S.A.) and 5% FCS at 37 °C in humidified atmosphere of 95% air–5% CO_2. The ^{51}Cr-labelled CRBCs were subsequently washed three times and adjusted to 2×10^5 cells/ml with RPMI 1640 medium containing 10% FCS.

NK Assay——In the experiment using K-562 cells as target cells, 100 μl of ^{51}Cr-labelled K-562 cell suspension (1×10^4 cells) and 100 μl of mononuclear cell suspension (3×10^5 cells) pretreated with IFN or control solution were added to each well of Nunc plastic tissue culture plates (U-bottomed, 96 wells) prior to incubation for 4 h at 37 °C in humidified atmosphere of 95% air–5% CO_2. In the experiments using A-549 and HMV-1 target cell, 100 μl of mononuclear cell suspension (3×10^5 cells) pretreated with IFN or control solution was added to each well containing monolayer tumor cells. These plates were incubated for 4 h under the conditions described above. The supernatants in the wells were separated by automated equipment (Titertek Supernatant Collection System; Flow Laboratories, Virginia, U.S.A.) for measurement of their radioactivities with a well-type gamma counter (Aloka JDC-751; Aloka, Tokyo, Japan).

ADCC Assay——In the experiment using CRBC cells, 50 μl of ^{51}Cr-labelled CRBC suspension (1×10^4 cells), 50 μl of a 1/2500 diluted solution of the immunoglobulin G (IgG) fraction of rabbit anti-CRBC antiserum (Fujizoki Pharmaceutical, Tokyo, Japan) and 100 μl of mononuclear cell suspension (3×10^5 cells) were added to each well of Nunc Plastic tissue culture plates, prior to incubation for 6 h. When A-549 or HMV-1 cells were used as target cells, 50 μl of mononuclear cell suspension (3×10^5 cells) and 50 μl of antibody solution prepared by 1/12.5 dilution of rabbit anti-A-549 or anti-HMV-1 antiserum were added to wells containing ^{51}Cr-labelled A-549 or HMV-1 cells. After a 4-h incubation, the radioactivities of the supernatants were measured by the same procedures as used in the NK assay. Non-specific cytotoxicity against tumor cells was not observed when heat-inactivated normal rabbit serum was used.

ADMC Assay——ADMC activity was determined by procedures similar to those used in ADCC assay except that 3×10^4 monolayer monocytes were used as effector cells in the latter experiment.

MTC Assay——A 100 μl aliquot of ^{125}I-labelled A-549 cells (5×10^4 cells) was added to monolayer monocytes (1×10^5 cells) in each well of Nunc plastic tissue culture plates. These plates were incubated for 72 h to determine the released radioactivities in the supernatants.

Calculation of Cytotoxicity——The following formula was used to calculate the percent specific lysis:

$$\% \text{ specific lysis} = \frac{\text{experimental cpm} - \text{SR cpm}}{\text{MR cpm} - \text{SR cpm}} \times 100$$

In this formula, MR cpm is the cpm of the supernatant obtained after lysis of ^{51}Cr- or ^{125}I-labelled target cells with 5% Triton X or with distilled water. SR cpm is the radioactivity spontaneously released from these target cells by the addition of medium in the absence of effector cells. Experimental cpm is the radioactivity released after effector-target cell reaction.

The value as a percentage of the control was calculated by using the following formula:

$$\% \text{ of control} = \frac{\% \text{ specific lysis at 10, 100 or 1000 unit/ml of IFN}}{\% \text{ specific lysis at 0 unit/ml of IFN}} \times 100$$

Results

Effect on NK Activity

Figure 1 shows the effects of recombinant human IFN-gamma (Met–Gln form), and natural human IFN-alpha and -beta on the NK activity of human mononuclear cells against K-562 cells. The augmentation of NK activity by IFN-gamma (Met–Gln form) was equivalent to those by natural IFN-alpha and -beta at 1000 unit/ml, but was somewhat inferior at the concentrations of 10 and 100 unit/ml. IFN-gamma (Met–Gln form) also augmented NK activity to 135%±8% of the control (mean±S. E. of three donors) at 5000 unit/ml (not determined for the natural IFNs), which is almost the same degree of augmentation as at 1000 unit/ml (Fig. 1).

Effect on ADCC Activity against CRBC

The effects of recombinant human IFN-gamma (Met–Gln form) and natural human IFN-alpha and -beta on the ADCC activity of human mononuclear cells against anti-CRBC IgG-coated CRBC are shown in Fig. 2. These IFNs augmented ADCC activity in

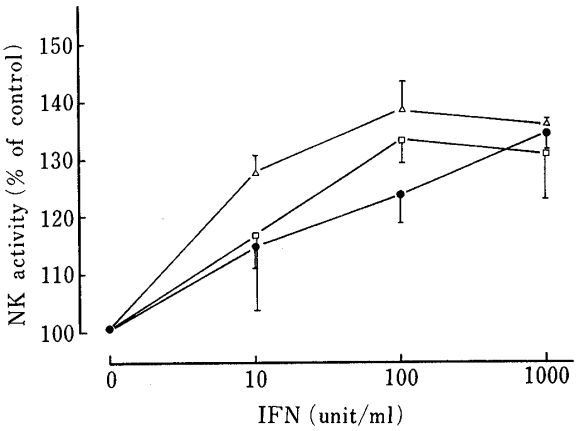

Fig. 1. Effects of IFNs on NK Activity against K-562 Cells

●, IFN-gamma; △, IFN-beta; □, IFN-alpha. Each point and vertical bar represent the mean± S.E. of three donors.

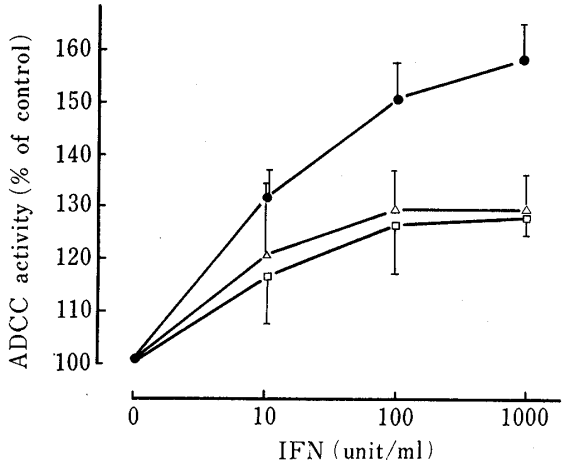

Fig. 2. Effects of IFNs on ADCC Activity against CRBC

●, IFN-gamma; △, IFN-beta; □, IFN-alpha. Each point and vertical bar represent the mean± S.E. of three donors.

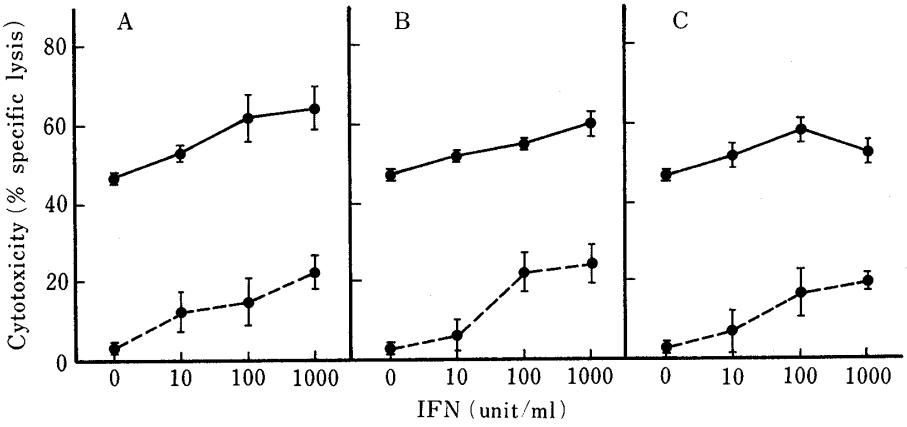

Fig. 3. Effects of IFNs on NK and ADCC Activities against HMV-1 Cells

A, IFN-gamma; B, IFN-beta; C, IFN-alpha; ●---●, NK activity; ●—●, ADCC activity.
Each point and vertical bar represent the mean±S.E. of three donors.

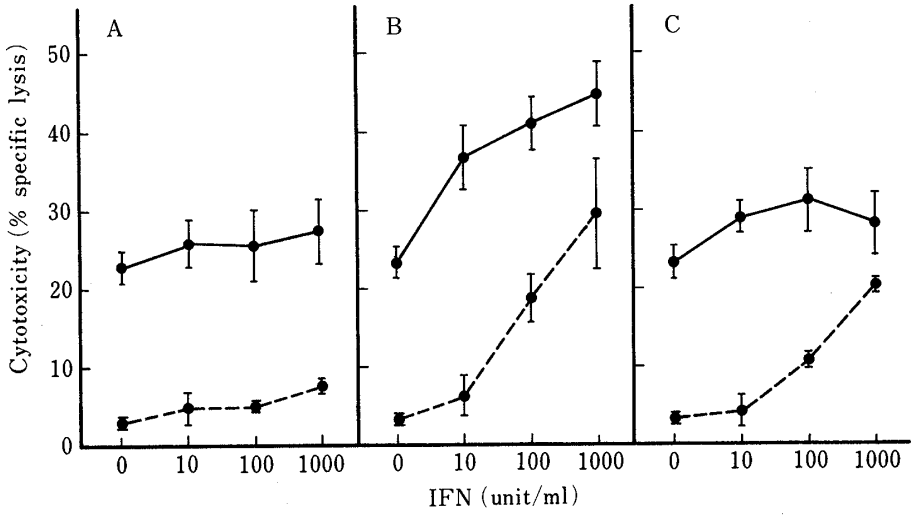

Fig. 4. Effects of IFNs on NK and ADCC Activities against A-549 Cells

A, IFN-gamma; B, IFN-beta; C, IFN-alpha; ●---●, NK activity; ●—●, ADCC activity.
Each point and vertical bar represent the mean ± S.E. of three donors.

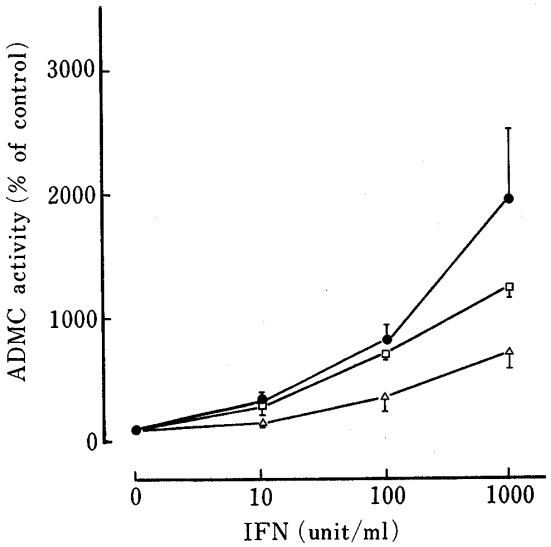

Fig. 5. Effects of IFNs on ADMC Activity

●, IFN-gamma; △, IFN-beta; □, IFN-alpha.
Each point and vertical bar represent the mean ± S.E. of three donors.

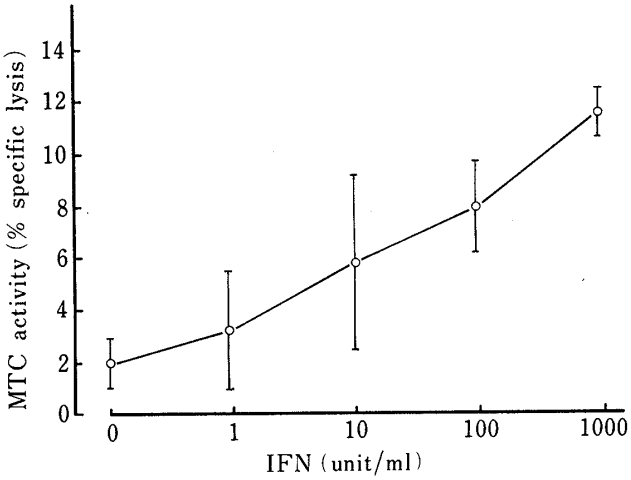

Fig. 6. Effect of Recombinant IFN-Gamma on MTC Activity

Each point and vertical bar represent the mean ± S.E. of three donors.

a dose-dependent fashion, and the augmentation by IFN-gamma (Met–Gln form) was superior to those by natural IFN-alpha and -beta at concentrations ranging from 10 to 1000 unit/ml.

Effects on NK and ADCC Activities against Tumor Cells

The effects of IFNs on NK and ADCC activities of human mononuclear cells against HMV-1 target cells were examined and the results are shown in Fig. 3. Recombinant human IFN-gamma (Met–Gln form) augmented NK activity in a dose-dependent fashion, and the effects were similar to those shown by natural human IFN-alpha and -beta. These IFNs equally augmented ADCC activity.

Figure 4 shows the effects of the IFNs on the NK and ADCC activities of human mononuclear cells against antibody-uncoated and -coated A-549 cells. In contrast to the

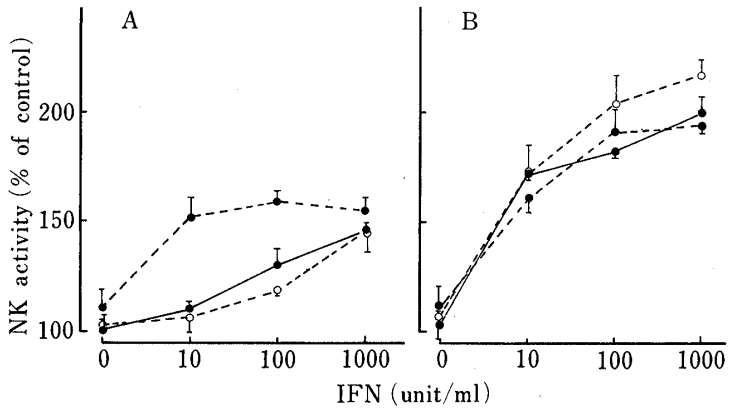

Fig. 7. Effects of Indomethacin and PGE_2 on NK-Augmentation by IFNs

A, IFN-gamma; B, IFN-beta; ●—●, IFN-treated; ●---●, IFN- and indomethacin-treated; ○---○, IFN-, indomethacin- and PGE_2-treated.
Each point and vertical bar represent the mean ± S.E. of three donors.

results obtained using HMV-1 cells, recombinant human IFN-gamma (Met–Gln form) did not clearly augment NK and ADCC activities, while natural human IFN-beta markedly augmented both NK and ADCC activities. A moderate augmentation of the activities was caused by natural human IFN-alpha treatment.

Effect on ADMC Activity against CRBC

Recombinant human IFN-gamma (Met–Gln form) and natural human IFN-alpha and -beta were examined for their augmenting effects on the ADMC activity of human monocytes against anti-CRBC IgG-coated CRBC. As shown in Fig. 5, IFN-gamma (Met–Gln form) markedly augmented ADMC activity in a dose-dependent manner. The augmentation was somewhat higher than that induced by natural human IFN-alpha at 1000 unit/ml, but their effects were similar to each other at 10 and 100 unit/ml. The effects observed with natural human IFN-beta were less marked than those with IFN-alpha and -gamma.

Effect on MTC Activity

Figure 6 shows the effects of recombinant human IFN-gamma (Met–Gln form) on MTC activity of human monocytes against A-549 cells. IFN-gamma (Met–Gln form) markedly augmented MTC activity in a dose-dependent manner.

Effects of Indomethacin and PGE_2 on the NK-Augmentation

To investigate the participation of PGs in the modulation of NK activity by IFNs, we examined the effects of indomethacin and PGE_2 on the augmentation of NK activity of human mononuclear cells against K-562 cells by recombinant human IFN-gamma (Met–Gln form) and natural human IFN-beta. As shown in Fig. 7A, combined addition of indomethacin (10^{-6}M) with IFN-gamma (Met–Gln form) resulted in further enhancement of the NK-augmentation as compared with that induced by IFN alone and further addition of PGE_2(10^{-8}M) completely reversed the effect of indomethacin. Coaddition of indomethacin and PGE_2 had no effect on the NK-augmentation induced by natural human IFN-beta (Fig. 7B).

Discussion

The results obtained in this study demonstrate that recombinant human IFN-gamma (Met–Gln form) does augment various cell-mediated cytotoxic activities, including NK, ADCC, ADMC and MTC activities, indicating that human immunomodulation caused by

recombinant human IFN-gamma may not require the N-terminal residue, Cys–Tyr–Cys.

The augmentation of NK activity against tumor cells and ADCC activity against antibody-coated tumor cells by IFN-gamma (Met–Gln form) was similar or somewhat inferior to that by natural human IFN-alpha and -beta (Figs. 1, 3 and 4). On the other hand, the augmentation of ADCC activity of mononuclear cells against anti-CRBC IgG-coated CRBC by IFN-gamma (Met–Gln form) was superior to that by IFN-alpha and -beta (Fig. 2). The disparity seen between these activities may be due to the difference of participating effector cells. In the ADCC reaction against sensitized tumor cells, the effector cells in our mononuclear cell preparation were considered to be K cells because it has been reported that the capacity to kill neoplastic cells in ADCC is low in immature monocytes.[8] On the other hand, in the ADCC reaction against anti-CRBC IgG-coated CRBC, effector cells are known to be both K cells and monocytes.[9] Therefore, we suppose that the prominent activity shown by IFN-gamma (Met–Gln form) in the ADCC reaction against anti-CRBC IgG-coated CRBC might be due to the more effective augmentation of monocytes in the mononuclear cell preparations by IFN-gamma (Met–Gln form). In fact, the augmentation seen in the ADMC activity of monocytes against anti-CRBC IgG-coated CRBC was more marked after the treatment with IFN-gamma (Met–Gln form) than IFN-alpha or -beta (Fig. 5). The augmentation of ADMC activity by IFN-gamma (Met–Gln form) may be attributed in part to the induction of the increased expression of Fc receptors on monocytes, as described in a separate report.[10] These results suggest that IFN-gamma (Met–Gln form) activates monocytes and macrophages rather than NK and K cells to kill tumor cells more effectively than IFN-alpha and -beta.

We found that IFN-gamma (Met–Gln form) augmented MTC activity (Fig. 6). The participation of contaminating NK cells in this MTC-augmentation may be negligible, because IFN-gamma (Met–Gln form) had a very weak effect on NK activity against the same target cells (Fig. 3). We also examined the effects of natural human IFN-alpha and -beta on MTC activity and the two natural IFNs apparently augmented the activity (data not shown). However, it is considered that the natural IFNs may have activated contaminating NK cells, not monocytes, because the IFNs markedly augmented NK activity against A-549 cells (Fig. 3).

Because of the possibility that PGs released from activated macrophages interfere with the cytotoxicity of NK cells, we examined the involvement of PGs in the augmentation of NK activity by IFNs. Treatment of the cells with indomethacin as well as IFN-gamma (Met–Gln form) or natural IFN-beta resulted in a clear enhancement of the NK-augmentation activity of IFN-gamma (Met–Gln form) but not IFN-beta. The enhancement was completely reversed by addition of PGE_2. These results suggest that PGs, or at least PGE_2, may be released from monocytes activated by IFN-gamma (Met–Gln form) and play a role as a suppressor during the manifestation of the NK augmentation effect. Our results obtained with IFN-beta are consistent with those reported by Dore-Duffy *et al.*[11] and Leung and Koren.[12] They have demonstrated that natural human IFN-beta inhibits PGE_2 synthesis in human monocytes and protects NK cells from suppression by PGE_2.

It would be interesting to investigate whether the immunomodulating activities of these IFNs observed in the present *in vitro* experiments reflect the *in vivo* situation. Due to difficulties of examination in humans using human IFNs, further experiments will be conducted in animals using animal IFNs to clarify the *in vivo* effects.

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