Augmentation of Antibody-Dependent Cellular Cytotoxicity of Polymorphonuclear Leukocytes by Interferon-Gamma: Mechanism Dependent on Enhancement of Fc Receptor Expression and Increased Release of Activated Oxygens

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(Received September 19, 1986)

The effect of recombinant human interferon (IFN)-gamma (Met-Gln form), having the same amino acid sequence as that of natural human IFN-gamma except for the N-terminal Met residue, on antibody-dependent cellular cytotoxic (ADCC) activity of human peripheral polymorphonuclear leukocytes (PMNs) against anti-chicken red blood cell (CRBC) immunoglobulin G (IgG)-coated CRBC was investigated comparatively with those of natural human IFN-alpha and -beta in vitro. IFN-gamma dramatically induced the ADCC activity at all doses tested when examined at a 1/10^4 dilution rate of the IgG, while no significant induction was observed with IFN-alpha or -beta. The Fc receptor (FcR) analysis by flow cytometry using fluorescein isothiocyanate-labelled (FITC)-anti-CRBC IgG revealed that IFN-gamma but not IFN-alpha or -beta remarkably increased the number of PMNs bearing FcR. By applying an in vitro measurement system for activated oxygen species using luminol solution, it was demonstrated that activated oxygen species were actually released from PMNs in the ADCC reaction. Further, inhibition experiments using three oxygen scavengers (superoxide dismutase, catalase and dimethyl sulfoxide) demonstrated the involvement of superoxide anion and hydroxy radical as cytolytic mediators in the PMN-ADCC activity induced by IFN-gamma.

These results indicate a much more potent PMN-activating ability of IFN-gamma than IFN-alpha or -beta and involvement of a mechanism dependent on the enhancement of FcR expression on the PMN surface and the increased release of superoxide anions and hydroxy radicals in the PMN-ADCC augmentation by IFN-gamma.

Keywords — interferon; polymorphonuclear leukocyte; antibody-dependent cellular cytotoxicity; Fc receptor; activated oxygen

Introduction

It is well known that polymorphonuclear leukocytes (PMNs) in human peripheral blood have Fc receptors (FcRs) on their surface and have antibody-dependent cellular cytotoxic (ADCC) activity against target cell sensitized with specific antibody. Many reports have described the interferon (IFN)-induced augmentation of various immunomodulating activities, including ADCC activities of human peripheral lymphocytes and monocytes. However, there are few reports dealing with the effects of IFNs on ADCC activity of PMNs. Hokland and Berg reported that human leukocyte IFN enhanced the ADCC activity of human PMNs, but Farr et al. found that human PMN functions including ADCC activity were unaffected by recombinant human IFN-alpha. Recently, Basham et al. have demonstrated that recombinant human IFN-gamma (Cys–Tyr–Cys–Gln form) having Cys–Tyr–Cys–Gln at the N-terminal augmented the PMN-ADCC activity more effectively than...
recombinant human IFN-alpha and -beta at a low dose.3c) Shalaby et al.3d) have also reported the augmentation of PMN-ADCC activity induced by recombinant human IFN-gamma (Met–Gln form) which has the same amino acid sequence as that of natural human IFN-gamma as elucidated by Rinderknecht et al.5) except for the N-terminal Met residue.3d) In spite of these studies, the mechanism of the IFN-gamma-induced augmentation is still unclear.

In the present study, we first attempted to compare the PMN-ADCC activity-augmenting ability of recombinant human IFN-gamma (Met–Gln form) with those of natural human IFN-alpha and -beta. IFN-gamma had a much stronger ability to induce PMN-ADCC activity than IFN-alpha or -beta at a low concentration of antibody. Further, to clarify the mechanism of the IFN-gamma-induced augmentation, we investigated the effects of these IFNs on the expression of FcR on human PMNs, because our previous studies had revealed that IFN-gamma augments the ADCC activity of human monocytes by increasing the number of monocytes bearing FcR.6) As expected, IFN-gamma but not IFN-alpha or -beta actually increased the number of PMNs bearing FcR.

Next, we attempted to identify cytolytic mediators participating in the PMN-ADCC reaction. Since it was reported that one of the cytolytic mediators involved in the PMN-ADCC reaction was superoxide anion (O2·−),7) enhancement of the production and release of O2·− by IFN-gamma is one possibility. To test this hypothesis, we tried to measure the amount of activated oxygen species released from PMNs during the PMN-ADCC reaction. The data indicate actual release of activated oxygen species in the ADCC reaction. Further, to elucidate the species of activated oxygen, we performed inhibition experiments using three scavengers of activated oxygen species. It was indicated that the cytolytic mediators involved were O2·− and hydroxy radical (·OH), and that IFN-gamma possibly augmented the PMN-ADCC activity by enhancing the production and release of these two species of activated oxygen.

**Experimental**

**Interferons**—Recombinant human IFN-gamma (specific activity: 2.72 × 10⁷ 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 × 10⁷ unit/mg protein) was prepared by the super induction method using human diploid fibroblasts in our laboratories as previously described.8) Natural human IFN-alpha (3.72 × 10⁶ unit/mg protein) was kindly provided by Dr. K. Cantell (Central Public Health Laboratory, Helsinki, Finland). The titer of each IFN represents the antiviral activity determined by the 50% cytopathic effect reduction method using FL cells and VSV or Sindbis virus.

**Preparation of PMNs**—Heparinized human peripheral blood (50 ml) obtained from a normal volunteer was mixed with 15 ml of saline containing 6% dextran, and the mixture was left for 60 min. The upper layer was removed and layered over Lymphoprep (Nyegaad, Oslo, Norway). After centrifugation for 30 min at 400 × g, a pellet containing PMNs was obtained. The pellet was washed with Dulbecco’s phosphate-buffered saline not containing Mg²⁺ and Ca²⁺ (Dulbecco PBS(−)). Contaminating red blood cells in the pellet were destroyed by adding Tris-buffer containing 0.83%, NH₄Cl, and PMNs were resuspended in RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan) containing heat-inactivated 10%, fetal calf serum (FCS; Commonwealth Serum Laboratories, Melbourne, Australia). The purity of PMNs in the suspension was 93%, as judged by Giemsa staining. The percentage of the PMNs which were able to phagocytose Immunobeads (BIO-RAD, Cal., U.S.A.) was 89%, in the suspension.

**Treatment of PMNs with IFNs**—For ADCC assay, 100 μl of PMN suspension containing from 2 × 10⁴ to 2 × 10⁵ cells in RPMI 1640 medium containing 10% FCS and 100 μl of IFN solution or control solution were mixed in each well of Nunc plastic tissue culture plates (U-bottomed, 96 wells; Inter Med, Denmark) prior to incubation at 37 °C for 20 h in humidified atmosphere of 95% air−5% CO₂. After the incubation, PMNs in wells were washed three times with RPMI 1640 medium containing 10% FCS and 100 μl of the same medium was added to each well.

For flow cytometry, 1 × 10⁶ PMNs in 1 ml of RPMI 1640 medium containing 10% FCS were treated with 1 ml of IFN solution or control solution in Falcon 60 × 15 mm plastic dishes (Becton Dickinson, Oxnard, U.S.A.). These dishes were incubated for 20 h under the same conditions as described above. At the end of this incubation period, PMNs were gently exfoliated by using a rubber policeman, and then washed with RPMI 1640 medium containing 10% FCS and suspended in the same medium.

For measurement of activated oxygens, 5 × 10⁵ PMNs in 250 μl of RPMI 1640 medium containing 10% FCS and
250 µl of IFN solution or control solution were added to flat-bottomed glass tubes (1 cm i.d. × 4.5 cm). These tubes were incubated for 20 h under the same conditions as described above. After the incubation, the supernatants were removed and 100 µl of Dulbecco PBS(−) was added to each tube.

The viability of the IFN-treated PMNs was > 99%, as evaluated by the trypan-blue exclusion test.

ADCC Assay — ADCC activity was measured by 51Cr-release assay using chicken red blood cells (CRBCs) as target cells. CRBCs were separated from heparinized fresh whole blood of White Leghorn chickens (female). After washing, 5 × 10⁷ CRBCs were incubated in 600 µl of RPMI 1640 medium containing 300 µCi of Na₂⁵¹CrO₄ (New England Nuclear, Boston, U.S.A.) and 5% FCS for 3 h at 37 °C in humidified atmosphere of 95% air−5% CO₂. These ⁵¹Cr-labelled CRBCs were subsequently washed three times and adjusted to the desired density with RPMI 1640 medium containing 10% FCS. Then 50 µl of ⁵¹Cr-labelled CRBC suspension (1 × 10⁶ cells) and 50 µl of antibody solution of 1/250 or 1/2500 diluted rabbit anti-CRBC IgG (Fujizoki Pharmaceutical, Tokyo, Japan) were added to the wells containing PMNs treated with IFNs or control solution. In the inhibition experiments on the ADCC reaction by activated oxygen scavengers, 10 µl of a solution containing 63 units of superoxide dismutase (SOD, Sigma, St. Louis, U.S.A.), 210 units of catalase (Sigma, St. Louis, U.S.A.), or 21 µmol of dimethyl sulfoxide (DMSO, Kishida Chemical, Osaka, Japan) was added to each well. These plates were incubated at 37 °C for 6 h under the same conditions as described above to separation of the supernatants by automated equipment (Titertek Supernatant Collection System; Flow Laboratories, Virginia, U.S.A.). Their radioactivities were measured by using a well-type gamma counter (Packard AUTO-GAMMA 5780, Packard Instrument, Downers Grove, Ill., U.S.A.).

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 was determined as cpm in the supernatant obtained after the lysis of ⁵¹Cr-labelled CRBCs with distilled water. SR cpm was determined as cpm released from ⁵¹Cr-labelled CRBCs incubated with RPMI 1640 medium containing 10% FCS alone in the absence of PMNs.

FcR Analysis by Flow Cytometry — FcR analyses were performed by the same method as described previously. 6) In brief, 1 × 10⁶ PMNs which had been treated with IFN or control solution were labelled with rabbit FITC-anti-CRBC IgG (Cappel Laboratories, Cochranville, U.S.A.). These labelled PMNs were analyzed by using a Cytofluorograf system 50H (Ortho Diagnostic Systems, Raritan, U.S.A.) or Spectrum III (Ortho Diagnostic Systems, Raritan, U.S.A.).

Measurement of Activated Oxygen Species in ADCC Reaction — Flat-bottomed glass tubes containing 5 × 10⁵ PMNs treated with IFN or control solution were preincubated at 37 °C for 2 min, and then 100 µl of luminol solution (0.56 mm in Dulbecco PBS(−)), 50 µl of 1/250 or 1/2500 diluted solution of rabbit anti-CRBC IgG and 50 µl of fresh CRBC suspension (1 × 10⁷ cells) were added to each tube. Chemiluminescence was measured by a luminescence analyzer (Picolite 6100; Packard Instrument, Downers Grove, Ill., U.S.A.) for 10 s every 1 min up to 15 min after the addition of these solutions.

Results

Effects of Anti-CRBC IgG and E/T Ratio on ADCC Activity

Figure 1A shows the effects of anti-CRBC IgG on the ADCC activity of PMNs treated with IFN-gamma and control solution. PMNs treated with control solution had a weak ADCC activity at the 1/10³ and 1/10² dilution rates of the IgG but no ADCC activity was found at the 1/10⁴ dilution rate. On the other hand, PMNs treated with IFN-gamma had strong ADCC activities at all dilution rates except the higher one, at which a very weak ADCC activity was noted.

Figure 1B shows the effects of E/T ratio on the ADCC activity of PMNs treated with IFN-gamma and control solution at the 1/10⁴ dilution rate. ADCC activities of PMNs treated with IFN-gamma were increased in proportion to the increase of E/T ratio, but not with control solution.

Comparison of ADCC-Augmenting Abilities among IFNs

Figure 2 shows the dose-dependent effects of IFN-gamma on the ADCC activity of PMNs compared with those of IFN-alpha and -beta. Both IFN-gamma and -beta but not IFN-alpha induced an ADCC activity, and the induction by IFN-gamma was more marked than that by IFN-beta regardless of the IFN dose. In particular, at 10 unit/ml, the ADCC
activity of PMNs induced by IFN-gamma was about 8-fold higher than that by IFN-beta. **Effects of IFNs on FcR Expression in PMNs**

Figure 3 shows the histograms of PMNs treated with IFN and control solution and fresh PMNs obtained by flow cytometry using FITC-anti-CRBC IgG. As shown in the figure the patterns of these histograms are very similar to each other, indicating that the overnight incubation of PMNs with the three IFNs and control solution induced no significant effect on the FcR expression on the PMN surface. On the other hand, the number of PMNs bearing
FcR was significantly increased by treatment with IFN-gamma but not IFN-alpha or -beta when compared with fresh and overnight controls, as shown in Fig. 4. The overnight incubation in control solution had no effect on the percentage of PMNs bearing FcR in total PMNs (Fig. 4).

**Effects of IFNs on Activated Oxygen Production in the ADCC Reaction**

The effects of IFNs on the production of activated oxygen species for 15 min after the initiation of the ADCC reaction are shown in Fig. 5. IFN-gamma significantly augmented the production more effectively than IFN-beta and -alpha. The significant augmentation induced by IFN-gamma persisted for 30 min (data not shown).

**Effects of Oxygen Scavengers on ADCC Augmentation by IFN-gamma**

Figure 6 shows the effects of three oxygen scavengers, i.e. SOD, catalase and DMSO, on the ADCC augmentation induced by IFN-gamma at a 1/10000 dilution rate of anti-CRBC IgG. The figure indicates significant inhibitory effects of SOD and DMSO on the augmentation, while catalase had no effect. DMSO but not SOD or catalase inhibited the ADCC
activity of control PMNs. Similar results were obtained at the 1/1000 dilution rate of the IgG (data not shown).

**Discussion**

The present study confirms that recombinant human IFN-gamma (Met–Gln form) augmented ADCC activity against anti-CRBC IgG-coated CRBC of human peripheral PMNs, as reported by Shalaby et al. 

Although Basham et al. have reported that recombinant human IFN-gamma (Cys–Tyr–Cys–Gln form) augmented the PMN-ADCC activity more effectively than IFN-alpha and -beta at low doses (1 and 100 unit/ml) but not at 500 unit/ml in the presence of a suboptimal concentration of the IgG, the present study showed that only IFN-gamma (Met–Gln form) significantly induced strong ADCC activity at high doses ranging from 10 to 1000 unit/ml, while IFN-alpha and -beta did not, when the dilution rate of the IgG was 1/10⁴ (Fig. 2). It is considered that this discrepancy may be caused by the difference of the concentration of the IgG used, not the difference in the form of recombinant IFN-gamma. Namely, it is likely that the amount of the IgG at the 1/10⁴ dilution rate in our system is smaller than that in their system, because the ADCC activity of control PMNs in their system was very high (50% lysis) whereas that in our system was very low (almost 0% lysis) as shown in Fig. 2. Therefore, we speculate that the suboptimal concentration of the IgG in their study may correspond to a lower dilution rate than the 1/10⁴ dilution rate used in our study. This speculation may be supported by the finding that IFN-gamma augmented the PMN-ADCC activity more effectively than IFN-alpha and -beta at low dose (10 unit/ml) when the dilution rate of the IgG was 1/10³ (data not shown). Taking into account these findings, it is conceivable that IFN-gamma is able to show a stronger inducing ability of PMN-ADCC activity against CRBC than IFN-alpha and -beta when examined at a very low concentration of anti-CRBC IgG which induces no PMN-ADCC activity in control PMNs.

The present study also demonstrates that IFN-gamma remarkably increased the number of PMNs bearing FcR binding to anti-CRBC IgG (Fig. 4), indicating that IFN-gamma induced or augmented the PMN-ADCC activity by increasing the number of PMNs bearing the FcR. Namely, the increase in FcR-bearing PMNs may in turn imply a substantial increase of the E/T ratio. The FcR-bearing PMNs were about 5% of control PMNs as shown in Fig. 4, which is in conflict with the results reported by Zighelboim et al., who gave a value of 56%. However, they also reported that the PMNs binding to non-aggregated rabbit IgG amounted to 8%, which is consistent with our results. We believe that the increasing effect of IFN-gamma on the number of FcR-bearing PMNs observed in this study is reliable because F(ab’)_2 of FITC-anti-CRBC IgG did not bind to PMNs (data not shown).

On the other hand, the PMN-ADCC augmenting abilities of IFN-alpha and -beta as mentioned above cannot be explained simply in terms of the number of FcR-bearing PMNs, because of the lack of increase of the number (Fig. 4). Since a recent report indicated that there are different IFN-receptors for IFN-gamma and for IFN-alpha and -beta on human cells, there may be different IFN receptors on human PMNs, and IFN-alpha and -beta may have different effects from IFN-gamma on PMNs. Recently, Philip and Epstein have reported that tumor necrosis factor mediated human monocyte cytotoxicity against human tumor cells induced by IFN-gamma but not IFN-alpha. Therefore, it is possible that different molecules may mediate the PMN-ADCC augmentation induced by IFN-alpha and -beta. In addition, the involvement of other mechanisms in the PMN-ADCC augmentation by IFNs, such as an enhancement of lytic ability of PMNs after binding to target cells or of recruitment of PMNs participating in the ADCC reaction, cannot be excluded.

The inhibition experiments using three oxygen scavengers indicated that O₂⁻ and hydroxy radical (·OH) play a role as cytolytic mediators in the expression of the ADCC activity of
PMNs treated with IFN-gamma (Fig. 6). Since SOD as well as dimethyl sulfoxide (DMSO) completely inhibited the ADCC augmentation induced by IFN-gamma (Fig. 6), it is unlikely that $O_2^-$ damaged CRBC independently of '$OH. Although it is generally known that '$OH is produced from $O_2^-$ and $H_2O_2$, $^{12}$ catalase had no effect on the ADCC augmentation (Fig. 6), which is consistent with the finding reported by Capsoni et al. that catalase did not inhibit PMN-ADCC activity against anti-CRBC IgG-coated CRBC. $^7$ Therefore, we speculate that only $O_2^-$ but not $H_2O_2$ may have been converted into '$OH, which acted cytotoxicity on CRBC. However, the conversion mechanism is unclear. On the contrary, our experiments indicated that only '$OH but not $O_2^-$ was the cytolytic mediator in the ADCC reaction of control PMNs (Fig. 6). In this case, '$OH may be generated from the prostaglandin-biosynthesis system$^{13}$ because SOD and catalase had no effect on the control PMN-ADCC reaction (Fig. 6). However, the results are in conflict with the finding reported by Capsoni et al. that the cytolytic mediator was $O_2^-$ in the control PMN-ADCC reaction. $^7$ The reason for the discrepancy is unknown, but the possibility that the cytolytic mediators may be different depending on the level of the ADCC activity cannot be excluded because the ADCC activity in their system was high (60% lysis) while that in our system was low (from 5% to 15% lysis) (Fig. 2).

There are several reports demonstrating that the mechanism of the PMN-ADCC reaction is oxygen-independent. For instance, Cordier and Revillard reported that phospholipase A$_2$ was a cytolytic mediator in the PMN-ADCC reaction using CRBC as target cells. $^{14}$ Recently, Dallegrì et al. have shown that PMNs released both activated oxygen species and phospholipase A$_2$ in the PMN-ADCC reaction using Raji cells as target cells, and pointed out the possibility that either of the two cytolytic mediators may become dominant depending on the experimental conditions. $^{15}$ Therefore, the participation of phospholipase A$_2$ in the PMN-ADCC reaction in our study is also possible, but the contribution of the enzyme to the augmentation by IFN-gamma may be slight because SOD and DMSO completely inhibited the augmentation (Fig. 6).

In conclusion, we have found in the present study (1) strong inducing ability of IFN-gamma for PMN-ADCC activity, (2) the mechanism of IFN-gamma-induced augmentation is dependent on enhancement of FcR expression and increased release of $O_2^-$ and '$OH and (3) weak or no effects of IFN-alpha and -beta on the PMN functions. Since the pharmacological significance of these PMN-modulating activities of IFN-gamma is unclear, further investigations in animals using animal IFN-gamma seem to be necessary.

Acknowledgments The authors thank Miss M. Kondoh and Mrs. K. Yamanaka for their skillful technical assistance. We are grateful to Dr. A. Kitai and Mr. N. Naruse for their encouragement and support of this study.

References


