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Effect of Interferon on Activated Oxygen Production in Human Monocytes *in Vitro*: Direct Measurement of Phagosomal Activated Oxygens by a New Method Using Luminol-Binding Microspheres

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We examined the effects of recombinant human interferon (IFN)-gamma, having Met–Gln at the N-terminal, on the *in vitro* production of phagosomal and extracellular activated oxygen species by human monocytes after phagocytosis and compared the results with those for natural human IFN-alpha and -beta. The phagosomal production was measured by a new method using luminol-binding microspheres and the extracellular production was measured by two conventional methods using luminol solution and opsonized zymosan or yeast particles. We also examined the effect of the IFN-gamma on the phagocytic activity of human monocytes by using luminol-binding microspheres. It was found that IFN-gamma remarkably augmented both phagosomal and extracellular production of activated oxygen species after phagocytosis in a dose-dependent fashion. However, IFN-alpha and -beta had a weak effect on the phagosomal production only at the highest dose tested. The natural IFNs also augmented moderately or slightly the extracellular production stimulated by opsonized zymosan but not opsonized yeast particles. IFN-gamma augmented the phagocytic activity of human monocytes. These results demonstrated that the human monocytes treated with the IFN-gamma actually produced a larger quantity of phagosomal activated oxygen species than those treated with the natural IFNs, and the extracellular production did not always reflect the phagosomal production.

Keywords——interferon; macrophage; activated oxygen; phagocytosis; phagosome

Introduction

The phagocytic-bacteriocidal activity of macrophages is very important in the self-defense system against bacterial infections. Namely, macrophages and leukocytes are able to phagocytose bacteria and surround them with an envelope of plasma membrane to form phagosomes, and then secrete activated oxygen species and lysosomal enzymes to kill and digest them.[1] Since interferon (IFN)-gamma has been identified as one of the macrophage-activating factors by many investigators,[2] it was reasonable to consider that IFN-gamma augment the phagocytic-bacteriocidal activity of macrophages. Nathan *et al.* have recently reported that among various cytokines including natural and recombinant human IFN-alpha and recombinant human IFN-beta, only recombinant human IFN-gamma, which has Cys–Tyr–Cys–Gln at the N-terminal,[3] remarkably enhanced both the H_2O_2 secretion and the antitoxoplasma activity of human macrophages.[4] Their study strongly suggests that increased production of activated oxygen species in the phagosomes is induced by IFN-gamma. However, they did not measure directly the amount of intraphagosomal activated oxygen species, since no suitable method was available.

In our previous studies, it was demonstrated that recombinant human IFN-gamma, which has the same amino acid sequence as natural human IFN-gamma except for the N-terminal residue of Met, activated various cell-mediated cytotoxicities and enhanced Fc receptor expression in human monocytes more effectively than natural human IFN-alpha and

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-beta.[5] It was expected that our IFN-gamma would also augment the phagocytic-bacteriocidal activity of human monocytes more effectively than these natural IFNs. Fortunately, Uchida *et al.* of our laboratories have recently developed a new method to measure quantitatively the amount of intraphagosomal activated oxygen species of macrophages by detecting the chemiluminescence of ingested luminol-binding microspheres.[6] Using the new method, we comparatively examined the effects of our IFN-gamma and natural human IFN-alpha and -beta on activated oxygen production in human monocyte phagosomes *in vitro*. The results obtained demonstrated that the human monocytes treated with our IFN-gamma actually produced a larger quantity of phagosomal activated oxygen species than those treated with the natural IFNs. To compare the results obtained by this new method with those by conventional methods,[7] we also examined the effects of these IFNs on the extracellular production of activated oxygen species by human monocytes stimulated by opsonized zymosan and yeast particles. These experiments revealed that the extracellular production did not always reflect the phagosomal production.

Experimental

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 in our laboratories by the superinduction method using human diploid foreskin fibroblasts as previously described.[8] 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 Human Monocytes——Blood from healthy volunteers was collected in heparinized syringes by venipuncture. The blood was diluted with an equal volume of Dulbecco's phosphate-buffered saline minus Mg^{++} and Ca^{++} (Dulbecco PBS(−)) and mononuclear cells were separated from the diluted blood solution by centrifugation for 30 min at $400 \times g$ on Lymphoprep (Nyegaard, Oslo, Norway). The interface cells, namely 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). Monocytes were separated from the mononuclear cell suspensions by using a macrophage separating kit (consisting of MSP-P as a macrophage separation plate and MSP-E as an exfoliation agent, Japan Immunoresearch Laboratories, Takasaki, Japan). The monocytes were washed three times with Dulbecco PBS(−) and suspended in RPMI 1640 medium containing 10% FCS. Monocyte recovery was >90% as determined by esterase and Giemsa staining methods.

IFN Treatment——Half a million monocytes 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 at 37 °C in a humidified atmosphere of 95% air–5% CO_2. After the incubation, the supernatants were removed and 100 μl of Dulbecco PBS(−) was added to each tube.

For measurement of phagocytic activity, 2×10^6 monocytes in 1 ml of RPMI 1640 medium containing 10% FCS and 1 ml of IFN-gamma solution (1000 unit/ml) were added to each well of a plastic tissue culture plate (flat-bottomed, 24-well; Linbro Scientific, Conn., U.S.A.). The plate was incubated for 20 h at 37 °C in a humidified atmosphere of 95% air–5% CO_2. After the incubation, the supernatants were removed and 500 μl of Dulbecco PBS(−) was added to each well. After exfoliation of the monocytes adhering to the well by using a rubber policeman, the monocyte suspension obtained was added to flat-bottomed glass tubes and these tubes were centrifuged at 1000 rpm for 1 min to remove 400 μl of the supernatants.

Preparation of Opsonized Yeast and Zymosan——Yeast particles (Oriental Yeast Co., Tokyo, Japan) were boiled for 30 min, then washed with Dulbecco PBS(−) until the supernatant was clear, and pelleted by centrifugation at 800 rpm for 8 min. The pelleted yeast particles (1×10^9 cells) were suspended in 1 ml of fresh human serum and incubated at 37 °C for 30 min. After the incubation, the opsonized yeast particles were washed twice and suspended in Dulbecco PBS(−).

To prepare opsonized zymosan, 7.5 mg of zymosan A (Sigma Chemical, St. Louis, U.S.A.) was suspended in and washed with Dulbecco PBS(−) to make the supernatant clear, and then pelleted by centrifugation at 800 rpm for 8 min. The pelleted zymosan was opsonized as described above.

Measurement of Phagosomal Activated Oxygen Species Using Luminol-Binding Microspheres——In order to

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measure the amount of activated oxygen species in phagosomes of monocytes after phagocytosis, we used the luminol-binding microsphere (2 μm in diameter) method reported by Uchida *et al.*[6] The principle of the method is to determine quantitatively activated oxygen species released in phagosomes of monocytes by measuring chemiluminescence originating from ingested and oxdized microsphere-bound luminol. In brief, flat-bottomed glass tubes containing 5×10^5 monocytes treated with IFN or control solution were preincubated at 37 °C for 2 min, and then 2.5×10^7 luminol-binding microspheres suspended in 10 μl of Dulbecco PBS(−) were added to each tube. Chemiluminescence was measured with a luminescence analyzer (Biocounter M2010, Lumac, Düsseldorf, West Germany) for 10 s every 1 min up to 15 min after the addition of the microspheres.

Measurement of Activated Oxygen Species Produced by Opsonized Yeast- and Zymosan-Stimulated Monocytes——Flat-bottomed glass tubes containing 5×10^5 monocytes treated with IFN or control solution were preincubated at 37 °C for 2 min, and then 50 μl of luminol solution (0.56 mM in Dulbecco PBS(−)) and 100 μl of opsonized yeast particle suspension (1.4×10^7) or opsonized zymosan suspension (2.5 mg/ml) were added to each tube. Chemiluminescence was measured with a luminescence analyzer (Biocounter M2010 or Picolite 6100, Packard Instrument, Downers Grove, U.S.A.) as described above.

Measurement of Phagocytic Activity——Flat-bottomed glass tubes containing 5×10^5 monocytes treated with IFN or control solution were preincubated at 37 °C for 2 min, and then 2.5×10^7 luminol-binding microspheres in 10 μl of Dulbecco PBS(−) were added to each tube prior to incubation for 10 min at 37 °C. After the incubation, 10 μl of 20-fold diluted Ziehl's solution (Nakarai Chemicals, Kyoto, Japan) was added to these tubes in order to stain the non-phagocytosed microspheres. The numbers of monocytes phagocytosed and non-phagocytosed microspheres in 10—20 visual fields chosen at random were counted under a microscope (400×).

Time-Courses of Activated Oxygen Production——All figures show the time-courses of activated oxygen production in human monocytes from a single donor. Similar data were obtained from two other donors (data not shown), but we did not calculate the mean values because there were minor but distinct individual differences between the patterns of the time-courses obtained from the three donors.

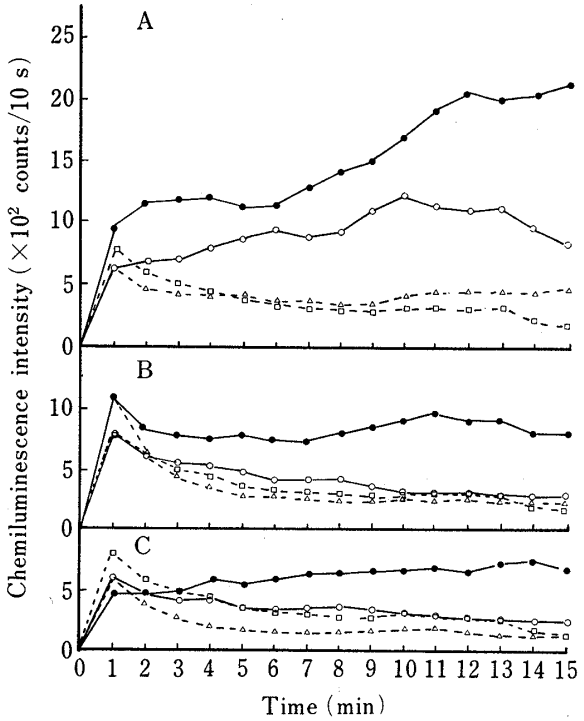

Fig. 1. Time-Courses of Phagosomal Production of Activated Oxygen Species by Human Monocytes Treated with IFNs

A, recombinant human IFN-gamma; B, natural human INF-beta; C, natural human IFN-alpha; △, 10 unit/ml; ○, 100 unit/ml; ●, 1000 unit/ml; □, control solution.

These data were obtained from a single donor.

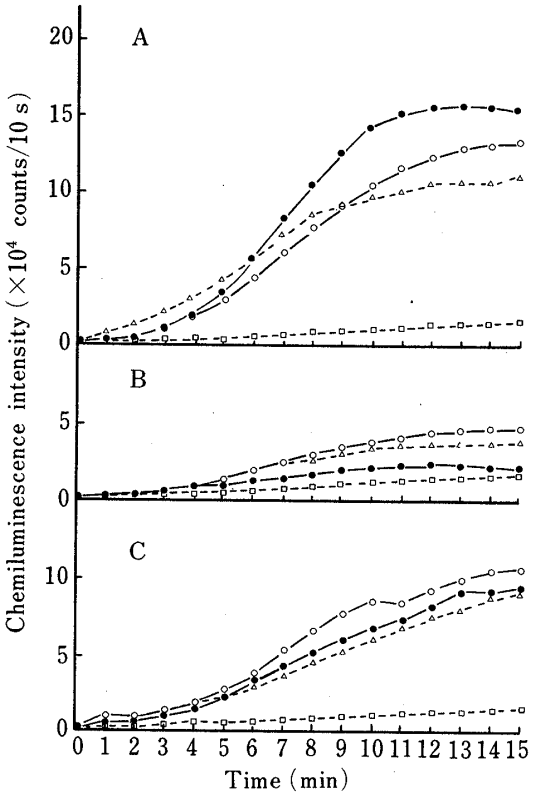

Fig. 2. Time-Courses of Extracellular Production of Activated Oxygen Species by Human Monocytes Treated with IFNs after Stimulation by Opsonized Zymosan

A, recombinant human IFN-gamma; B, natural human IFN-beta; C, natural human IFN-alpha; △, 10 unit/ml; ○, 100 unit/ml; ●, 1000 unit/ml; □, control solution.

These data were obtained from a single donor.

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Results

Effects of IFNs on Phagosomal Activated Oxygen Production by Human Monocytes

Figure 1 shows the time-courses of phagosomal production of activated oxygen species after addition of luminol-binding microspheres to human monocytes pretreated with recombinant IFN-gamma, natural IFN-alpha, -beta or control solution for 20 h. As shown in Fig. 1A, recombinant IFN-gamma markedly increased the phagosomal production of activated oxygens by monocytes at concentrations of 100 and 1000 unit/ml but not at 10 unit/ml. On the other hand, natural IFN-alpha and -beta apparently increased the production only at 1000 unit/ml, the level of production in this case being higher than the control but less than that shown by the same concentration of IFN-gamma (Fig. 1B and 1C).

Effects of IFNs on Opsonized Zymosan-Stimulated Production of Activated Oxygen Species by Human Monocytes

The time-courses of opsonized zymosan-stimulated production of activated oxygen species by human monocytes pretreated with IFN or control solution are shown in Fig. 2. Recombinant IFN-gamma at concentrations of 10, 100 and 1000 unit/ml markedly increased the production in a time-dependent manner, reaching a plateau from 9 to 15 min after addition of opsonized zymosan (Fig. 2A). On the other hand, natural IFN-beta only slightly increased the production regardless of the concentration tested (Fig. 2B). Natural IFN-alpha also increased the production without dose-dependency (Fig. 2C).

Effects of IFNs on Opsonized Yeast Particle-Stimulated Production of Activated Oxygen Species by Human Monocytes

We also examined the time-courses of opsonized yeast particle-stimulated production of activated oxygen species by monocytes pretreated with IFN or control solution. Since the data obtained from these experiments are similar to those shown in Fig. 2, they are not illustrated, but may be summarized as follows. Recombinant IFN-gamma time-dependently increased the production of activated oxygen species at concentrations of 100 and 1000 unit/ml and the peak of the activity was observed at about 10 min after the addition of opsonized yeast particles. At 10 unit/ml, the effect was similar to the control. Natural IFN-beta and natural IFN-alpha at 100 and 1000 unit/ml gave results similar to or slightly less than

TABLE I. Effects of Recombinant Human IFN-Gamma and Natural Human IFN-Alpha and -Beta on Phagosomal and Extracellular Production of Activated Oxygen Species by Human Monocytes

Treatment	IFN titer (unit/ml)	Total intensity of chemiluminescence, counts/15 min		
		Luminol-binding microspheres ($\times 10^5$)	Opsonized yeast particles ($\times 10^7$)	Opsonized zymosan ($\times 10^7$)
Control	0	3.1±0.2 (100)	13.0±2.6 (100)	1.1±0.2 (100)
Recombinant IFN-gamma	10	3.5±0.5 (113)	10.7±2.8 (82)	5.5±0.4 (500)
	100	7.3±0.5 (235)	16.0±1.5 (123)	5.8±0.7 (527)
	1000	13.2±2.6 (426)	15.7±1.2 (121)	9.0±1.1 (818)
Natural IFN-beta	10	3.4±0.5 (110)	13.7±2.3 (105)	1.7±0.2 (155)
	100	3.5±0.2 (113)	6.9±1.6 (53)	2.0±0.2 (182)
	1000	8.0±1.4 (258)	6.3±0.7 (48)	2.7±0.9 (245)
Natural IFN-alpha	10	1.9±0.2 (61)	4.3±1.3 (33)	4.1±1.0 (373)
	100	2.9±0.3 (94)	10.2±1.3 (78)	3.9±0.8 (355)
	1000	4.3±0.7 (139)	8.1±2.2 (62)	3.2±0.7 (291)

Each value represents the mean±S.E. of three donors. The figures in parentheses indicate % of control.

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TABLE II. Effect of Recombinant Human IFN-Gamma on Phagocytic Capacity of Human Peripheral Blood Monocytes Determined by Using Luminol-Binding Microspheres and Microscopy

Treatment	Cell number of		
	Total monocytes counted	Monocytes with ingested microspheres	Monocytes without ingested microspheres
Control	154±2.2 (100)	84±10 (54)	71±9.5 (46)
Recombinant IFN-gamma (1000 unit/ml)	156±3.8 (100)	111±9.2 (70)	47±4.0 (30)

Each value represents the mean±S.E. of three donors. Figures in parentheses indicate % total monocytes counted.

the control. At 10 unit/ml, IFN-beta also gave a similar result to the control, but no chemiluminescence was detectable in the case of monocytes treated with IFN-alpha at 10 unit/ml.

Comparison of IFN-Induced Augmentation of Activated Oxygen Production by Human Monocytes

Table I summarizes the mean values of the cumulative chemiluminescence intensity for phagosomal and extracellular production of activated oxygen species in 15 min by IFN-treated and untreated monocytes obtained from three donors. Recombinant IFN-gamma dose-dependently augmented the cumulative phagosomal activated oxygen production and the augmentation was roughly ten times more effective than that by natural IFN-beta when compared on the basis of antiviral activity. Natural IFN-alpha decreased the cumulative production at 10 and 100 unit/ml but slightly increased it at 1000 unit/ml when compared with the control treatment. On the other hand, recombinant IFN-gamma only slightly increased cumulative extracellular activated oxygen production when stimulated by opsonized yeast particles at concentrations of 100 and 1000 unit/ml but decreased it slightly at 10 unit/ml; natural IFN-beta markedly decreased the cumulative production at 100 and 1000 unit/ml and natural IFN-alpha also decreased it at 10, 100 and 1000 unit/ml. Recombinant IFN-gamma markedly increased the cumulative extracellular activated oxygen production with apparent dose-dependency when stimulated by opsonized zymosan; natural IFN-alpha and -beta also increased the cumulative production but were less effective than IFN-gamma.

Effect of IFN-Gamma on Phagocytic Activity of Monocytes

To determine the effect of IFN-gamma on the phagocytic activity of human monocytes, the number of monocytes phagocytosed and the number of non-phagocytosed microspheres were counted after a 20 h treatment with 1000 unit/ml of the IFN. As shown in Table II, the ratio of phagocytosed monocytes per total monocytes counted was 70% for IFN-gamma-treated monocytes and 54% for control solution-treated monocytes. The results indicate an apparent enhancement of phagocytosis by the IFN-gamma treatment.

Discussion

The present *in vitro* study demonstrates that recombinant human IFN-gamma, having Met–Gln at the N-terminal, augmented the phagocytosis of luminol-binding microspheres and the production of phagosomal and extracellular activated oxygen species in human monocytes more effectively than natural human IFN-alpha and -beta. The IFN-induced augmentation of the phagocytic-bacteriocidal activity of macrophages suggested on the basis of previous *in vitro* studies, which demonstrated that murine IFN augmented the non-specific

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phagocytosis of carbon particles and latex beads by mouse macrophages, was confirmed by later *in vitro* studies demonstrating a mouse IFN-induced increase of the uptake of *E. coli* as well as of the number of mouse macrophages engaged in phagocytosis and the average number of bacteria ingested per macrophage.[9] Therefore, it is expected that human monocytes treated with the IFN-gamma may phagocytose and digest microorganisms more effectively than those treated with IFN-alpha and -beta *in vitro*, as reported in the case of recombinant human IFN-gamma having Cys–Tyr–Cys–Gln at the N-terminal.[4] Kinderlen *et al.* have recently reported that recombinant murine IFN-gamma protected mice *in vivo* from the intracellular bacterial pathogen *Listeria monocytogenes* in a local as well as in a systemic infection model.[10] By analogy with the present results using recombinant human IFN-gamma and human monocytes, it is likely that the murine IFN-gamma eliminated the pathogens in murine monocytes by augmenting the production of phagosomal activated oxygen species. Taking these results into consideration, it is suspected that recombinant human IFN-gamma, having Met–Gln at the N-terminal, may induce much stronger phagocytic-bacteriocidal activity mediated by human macrophages than human IFN-alpha or -beta *in vivo*.

The three systems used for measurement of activated oxygen species produced by human monocytes after stimulation gave different results for IFN-gamma, -alpha and -beta treatments. Since it is well known that luminol is converted to an excited aminophthalate ion in the presence of various activated oxygen species including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxy radical ($\cdot OH$) and singlet oxygen ($^1O_2$), and this reaction emits blue light,[7b] we detected various activated oxygen species produced by IFN-treated monocytes in the three test systems. It is likely that each IFN has different effects on the production of some or all of these oxygens because several pathways of activated oxygen production after phagocytosis have been proposed. Boraschi *et al.* reported that natural mouse IFN-beta significantly reduced the release of O_2^- and H_2O_2 from mouse macrophages in a dose-dependent fashion.[11] As described above, Nathan *et al.* showed that recombinant human IFN-beta slightly enhanced the H_2O_2-release by human macrophages, but natural human IFN-alpha did not.[4] Recently, Ito *et al.* have reported that natural mouse IFN-alpha enhanced chemiluminescence and $\cdot OH$ production but did not change O_2^- and H_2O_2 levels in mouse macrophages.[12] Since a recent report indicated that there are different IFN receptors for IFN-gamma and for IFN-alpha and -beta on human cells,[13] there may be different IFN receptors on human monocytes. Therefore, it is not unexpected that the three IFNs showed different effects on the production of these oxygen species. However, the mechanisms of the reducing effects of the natural IFNs on the activated oxygen productions under some conditions shown in Table I are still unclear. Although it is interesting of the different mechanisms of the augmentation and suppression in activated oxygen production induced by these IFNs, further investigations will be required to make them clear.

The present study has shown that the new method using luminol-binding microspheres is a useful and easy method for direct examination of the effect of a macrophage-modulating agent on phagosomal activated oxygen production *in vitro*. However, there are still many problems which should be resolved. For example, it is unclear why a transient peak appears immediately after addition of the microspheres to monocytes (Fig. 1). Although it is very difficult to examine in detail the phagocytic process of the microspheres by monocytes, further experiments should cast more light on these problems.

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