# Effect of interferon-$\gamma$ on human neutrophil chemiluminescence

Masumi Aoki, Yasuo Ono and Otohiko Kunii
The Second Department of Internal Medicine Teikyo University School of Medicine,
2-11-1 Kaga, Itabashi-ku, Tokyo 173, Japan

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We studied the effect of recombinant human interferon-$\gamma$ (IFN-$\gamma$) on the oxygen metabolite generation of human neutrophils *in vitro*. The incubation of whole blood with 1,000 or 10,000 units/ml of IFN-$\gamma$ for 4 hours at 37°C significantly enhanced luminol-dependent chemiluminescence (CL) stimulated by non-opsonized zymosan, phorbol myristate acetate (PMA) or *Pseudomonas aeruginosa* Nc-5. Four hours' incubation of neutrophils with IFN-$\gamma$ enhanced CL. Preincubating neutrophils with 1,000 units/ml of IFN-$\gamma$ for 4 hours resulted in a 1.16-fold increase in zymosan-induced CL and a 1.75-fold increase in *P. aeruginosa*-induced CL. However, IFN-$\gamma$ alone did not stimulate neutrophil CL. There was no significant enhancement during a 2 hours' preincubation with IFN-$\gamma$. Luminol-binding microsphere (lumispheres)-induced CL of neutrophils was also enhanced after incubation with 1,000 units/ml of IFN-$\gamma$ for 4 hours. In addition the effect of IFN-$\gamma$ on the neutrophils obtained from elderly patients was evaluated. An increase in CL comparable to that obtained from healthy young adults was observed. These findings suggest that IFN-$\gamma$ activates neutrophil function and may be clinically useful in immunosupportive therapy for intractable infections in elderly patients.

**Key words**: IFN-$\gamma$, Chemiluminescence, human neutrophils

Interferon-$\gamma$ (IFN-$\gamma$) activates monocytes and macrophages[1]. Less focus has been given to the modulation of neutrophils by IFN-$\gamma$. It has been reported that neutrophils primed by IFN-$\gamma$ have enhanced functional activity[2~4] However, in some cases, contradictory results have been reported[5~7]

Measurement of chemiluminescence (CL) is thought to be a reliable tool with which to study the generation of oxidative metabolites in inflammatory cells. In this study, using CL, we examined the effect of recombinant human IFN-$\gamma$ (IFN-$\gamma$) *in vitro* on the respiratory burst of neutrophils obtained from healthy young adults and elderly patients, and speculated on the use of IFN-$\gamma$ as an immunotherapeutic agent.

## Materials and Methods

### Subjects

Eighteen healthy volunteers (10 mens, 8 womens, median age 29.2 years, range 25—36) and nine elderly patients admitted to Teikyo University Hospital (4 mens, 5 womens, median age 78.8 years, range 70—89) were studied. All elderly patients had been admitted with neurologic or heart diseases without acute clinical conditions, glucose intolerance or elevation of serum creatinine levels.

### Chemicals and media

Dulbecco's modified Eagle's medium (Nissui, Tokyo) containing 25 mM HEPES and L-glutamin 0.3 g/l at pH 7.4 (MEM) was used to dilute samples. Luminol (Tokyo Kasei Kogyo, Tokyo) and zymosan (Sigma Chemical Co.) were dissolved in phosphate buffered saline (PBS) at 20 $\mu$g/ml and 25 mg/ml, respectively. Phorbol myristate acetate (Sigma Chemical Co.) was dissolved in dimethyl sulfoxide (DMSO) at 100 $\mu$g/ml. Recombinant human interferon-$\gamma$ (IFN-$\gamma$) was kindly provided by Shionogi Pharmaceuticals, Osaka, Japan, and lumisphers were purchased from TORAY Industries

Inc., Tokyo, Japan.

**Isolation and purification of neutrophils**

Neutrophils were prepared from heparinized venous blood (10 IU/ml blood) using mono-poly resolving medium (M-PRM, Flow Labs., Scotland). Blood (3.5—7 ml) was carefully overlaid on M-PRM (3 ml), and centrifuged at 300×g for 30 minutes at room temperature. The neutrophil layer was withdrawn, washed once with MEM, and resuspended in MEM.

**Growth of bacteria**

*Pseudomonas aeruginosa* Nc-5 was grown overnight on nutrient heart infusion broth at 37°C. Cells were then washed twice and resuspended in MEM to a concentration of about $10^9$ cells/ml.

**Incubation with IFN-γ**

Purified IFN-γ was diluted in sterile water and stored in portions at concentrations of 1,000,000 units/ml at −80°C; aliquots were not re-frozen after thawing. An aliquot was diluted with normal saline to a final concentration of 10,000 or 100,000 units/ml. Whole blood or neutrophils ($5\times10^5$/ml) were incubated with various concentrations of IFN-γ at 37°C for various lengths of time prior to assay. In all experiments, whole blood or neutrophil suspensions incubated for identical periods in the abscence of IFN-γ served as controls.

**Chemiluminescence measurements**

Chemiluminescence (CL) was measured using a Biolumat LB 9505 (Berthold Co., Germany) at 37 °C for 30 minutes.

(1) Luminol-dependent CL assay: (i) Whole blood CL: 0.1 ml of heparinized whole blood was added to round-bottomed tubes followed by the addition of 0.96 ml of MEM containing 20 μl of luminol and incubated at 37°C in the presence or absence of IFN-γ (10 μl). Non-opsonized zymosan (500 μg, 20 μl), PMA (0.5 μg, 5 μl) or *P. aeruginosa* Nc-5 ($10^8$ cells, 100 μl) was then added and CL was measured. (ii) Neutrophil CL: The neutrophil concentration was $5\times10^5$ cells/ml. CL was assayed in the same way as described in (i). In the *P. aeruginosa*-induced CL assay, $2\times10^7$ bacterial cells were added to samples containing 10 μl of human pooled serum.

(2) Lumisphere-induced CL assay: Samples containing $5\times10^5$ neutrophils were incubated, then 20 μl of lumispheres were added and CL was measured.

**Data presentation**

The integral CL over the whole period (30 minutes) was calculatd and IFN-γ-incubated cells were compared to IFN-γ-nonincubated (control) cells. Results are expressed as increased rate of the integral CL, that is the ratio of CL of IFN-γ-incubated cells to that of the control.

**Statistical analysis**

The statistical significance of differences between results was calculated by Student's *t*-test.

## Results

Stimulus-induced CL in whole blood obtained from healthy young adults was increased compared with control values when whole blood was tested after 4 hours' incubation in the presence of more than 1,000 units/ml of IFN-γ. During a 2 hours' incubation under the same conditions, there was no significant enhancement. As shown in Fig. 1, preincubating whole blood with 10,000 units/ml of IFN-γ for 4 hours resulted in 1.10-1.13- and 1.53-fold increases in zymosan-, PMA- and *P. aeruginosa*-induced CL, respectively. The CL response to 1,000 and 10,000 units/ml of IFN-γ treated neutrophils was similar. A dose-dependent response of luminol-enhanced CL was observed when neutrophils were incubated with IFN-rat various concentrations (10-1,000 units/ml) for 4 hours prior to the addition of *P. aeruginosa* (data not shown). Four hours' incubation of neutrophils with 1,000 units/ml of IFN-γ enhanced CL 1.75 fold (Fig. 2).

Preincubation of neutrophils with IFN-γ for 4 hours resulted in a 1.16-fold increase in zymosan-induced CL (Fig. 2). Significant enhancement was not observed with PMA, which differed from the results of whole blood CL, but the peak time was slightly reduced (Figs. 2, 3).

Lumisphere-stimulated CL was also enhanced after incubating neutrophils with 1,000 units/ml of IFN-γ for 4 hours (Fig. 4).

The addition of only IFN-γ did not stimulate neutrophil CL (data not shown). Also, during a 2-hour preincubation with IFN-γ.

Furthermore, comparing the effects of IFN-γ on

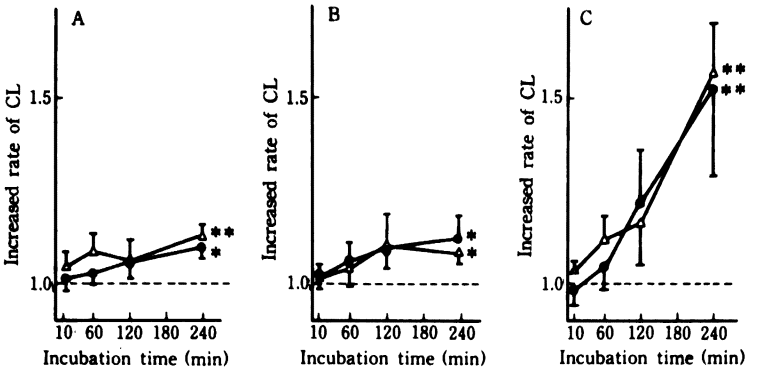


Fig. 1. Effect of IFN-γ on luminol-dependent whole blood CL.
Whole blood was incubated with 1,000 units/ml (△——△) or 10,000 units/ml (●——●) of IFN-γ. CL induced by zymosan (A), PMA (B) and *Pseudomonas aeruginosa* (C) was measured. Data presented are mean values ± standard errors of 6-16 separate experiments.
*$p<0.05$, **$p<0.01$

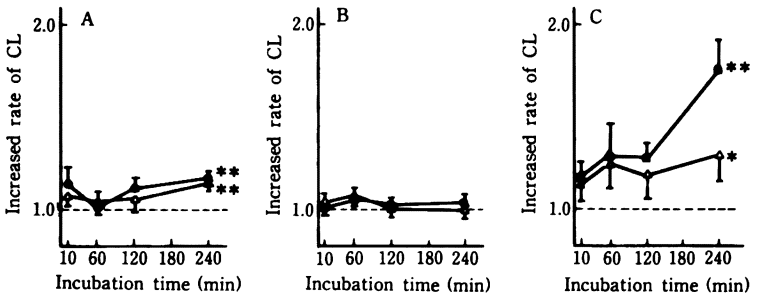


Fig. 2. Effect of IFN-γ on luminol-dependent neutrophil CL.
Neutrophil suspensions were incubated with 100 units/ml (△——△) or 1,000 units/ml (●——●) of IFN-γ. CL induced by zymosan (A), PMA (B) and *Pseudomonas aeruginosa* (C) was measured. Data presented are mean values ± standard errors of 5-18 separate experiments.
*$p<0.05$, **$p<0.01$

neutrophil CL obtained from healthy young adults with those from elderly patients (CL was assayed in the same way), we found that the increased rate of CL in the latter was slightly higher than that in the former, but the difference was not statistically significant (Fig. 5).

## Discussion

The results presented in this report demonstrate that preincubating neutrophils with IFN-γ for 4 hours increased their ability to generate reactive oxygen. Previous studies by other investigators have shown that IFN-γ enhances some neutrophil functions such as oxygen metabolism, phagocytic activities and fungal killing[2~4,8~12]. However, some reports on IFN-γ have been conflicting[5~7]. This study, using CL, demonstrated that IFN-γ activates neutrophil function to generate oxygen metabolites during the phagocytosis of non-opson-

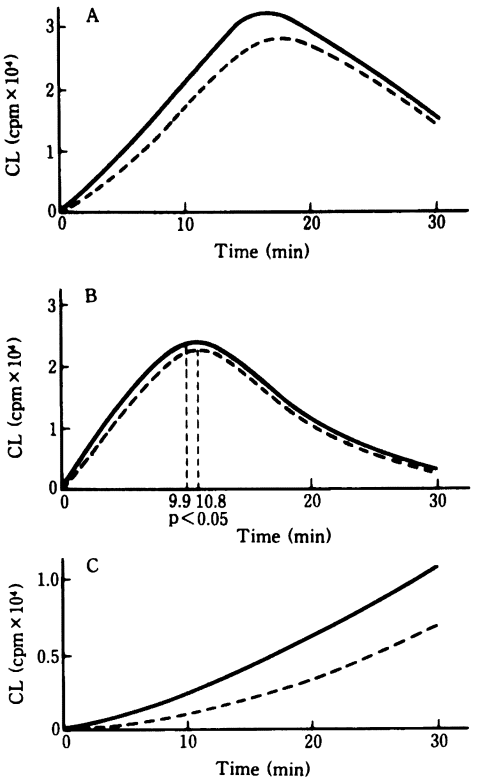


Fig. 3. Time course of IFN-$\gamma$ enhancement of luminol-dependent CL.
Neutrophil suspensions were incubated for 4 hours in the presence (——) or abscence (·····) of 1,000 units/ml of IFN-$\gamma$. After the addition of zymosan (A), PMA (B) and *Pseudomonas aeruginosa* (C), CL traces were recorded. Data presented are mean values obtained from 12 separate experiments.
cpm; counts per minute

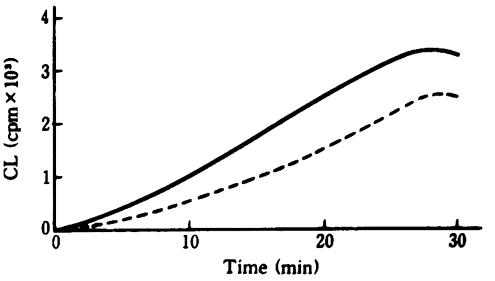


Fig. 4. Effect of IFN-$\gamma$ on lumisphere-induced neutrophil CL.
Neutrophil suspensions were incubated for 4 hours in the presence (——) or abscence (·····) of 1,000 units/ml of IFN-$\gamma$. Data presented are mean values obtained from 7 separate experiments.
cpm; counts per minute

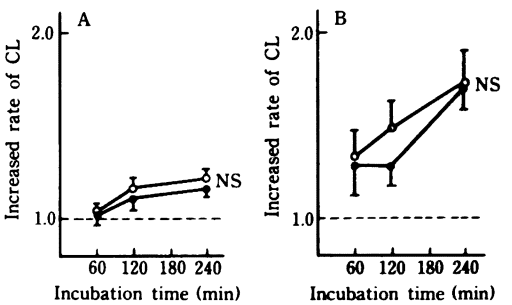


Fig. 5. Comparison of effects of IFN-$\gamma$ on the luminol-dependent neutrophil CL obtained from healthy young adults and elderly patients.
Neutrophil suspensions ($5\times10^5$/ml) were incubated with 1,000 units/ml of IFN-$\gamma$. CL induced by zymosan (A) and *Pseudomonas aeruginosa* (B) was measured. Data presented are mean values ± standard errors from 6-18 experiments in healthy young adults (●——●) and from 6-8 experiments in elderly patients (○——○).
NS; not significant

ized zymosan and *P. aeruginosa*. Our results are similar to those of Edwards *et al*[13], who reported that the preincubation of neutrophils with IFN-$\gamma$ increased luminol-dependent CL induced by preopsonized *Staphylococcus aureus* and enhanced the initial rate of bacterial killing.

We also demonstrated that IFN-$\gamma$ increased intracellular reactive oxygen levels during phagocytosis of luminol-binding microspheres (lumispheres).

The addition of IFN-$\gamma$ did not stimulate neutrophil CL *per se*, and IFN-$\gamma$ required a longer incubation time to exert an effect than either colony stimulating factor (CSF) or tumor necrosis factor (TNF). These results agree with those of Berton *et al.*[14] and Perussia *et al.*[15], who showed that incubation of neutrophils with IFN-$\gamma$ for several hours was necessary for the potentiation of superoxide

generation or CL response. The mechanism of action of IFN-γ may differ from that of other cytokines. It is possible that the effect of IFN-γ depends on RNA and *de novo* protein synthesis, increased expression of high-affinity receptors (FcR high) on the neutrophil surface or alteration of the NADPH-oxidase system[14~17]. However, the molecular basis of the mechanism has yet to be sufficiently clarified. Additional studies are necessary to elucidate the intracellular mechanisms underlying this increased neutrophil function.

Little is known about the impact of aging on the function of IFN-γ. It has been reported that IFN production is decreased in the elderly[18,19], but this study showed that IFN-γ-induced enhancement of neutrophil CL response in the elderly was similar to that in healthy young adults. Recently, the clinical efficacy of recombinant IFN-γ in chronic granulomatous disease (CGD), in which the ability of phagocytic cells to generate reactive oxygen is lacking or markedly impaired, has been recognized[20]. This finding was based on studies showing that rIFN-γ increased the superoxide production and bactericidal activity of phagocytes from patients with CGD[21,22]. In the elderly, reduction in neutrophil function, such as superoxide production, phagocytic activity and bactericidal capacity, has been reported[22,23]. Our findings suggest that IFN-γ activates neutrophil function (reactive oxygen production) and may be clinically useful in the control of intractable infection (e.g., mediated by *P. aeruginosa*) in elderly patients when IFN-γ production is decreased.

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## Recombinant human INF-γ のヒト好中球 Chemiluminescence におよぼす影響

青木ますみ・斧　康雄・国井　乙彦
帝京大学医学部第二内科*

我々は，ヒト好中球の活性酸素産生におよぼす recombinant human interferon-γ (IFN-γ) の影響を *in vitro* で検討した。全血を 1,000 または 10,000 units/ml の IFN-γ とともに 37°C で 4 時間保温すると，非オプソニン化 zymosan, phorbol myristate acetate (PMA) または *Pseudomonas aeruginosa* Nc-5 刺激後の luminol 依存性 chemiluminescence (CL) が有意に増強した。分離好中球と IFN-γ を 4 時間保温した場合も CL 反応を増強した。すなわち，好中球を IFN-γ 1,000 units/ml と 4 時間保温後，zymosan 刺激で 1.16 倍，*P. aeruginosa* 刺激で 1.75 倍の有意な増強効果がみられた。しかし，IFN-γ 自体は好中球 CL を誘導しなかった。また，2 時間以内の保温時間では有意な増強効果はみられなかった。Luminol-binding microsphere (lumisfere) を用いた好中球内 CL 反応においても，IFN-γ 1,000 units/ml と 4 時間保温後に有意な増強がみられた。さらに，高齢患者の好中球におよぼす IFN-γ の影響についても検討したところ，健康成人と同様の CL の増強がみられた。これらの成績から，IFN-γ が好中球機能を活性化することが示され，臨床的に，高齢患者の難治性感染症における免疫補助療法として有用となる可能性が示唆された。

* 東京都板橋区加賀 2-11-1