Gamma Interferon Enhances the Killing of *Staphylococcus aureus* by Human Neutrophils

By STEVEN W. EDWARDS,* JANE E. SAY AND VALERIE HUGHES

Department of Biochemistry, University of Liverpool, PO Box 147, Liverpool L69 3BX, UK

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The effect of purified human interferon- γ on the responsiveness of human neutrophils was investigated. Pre-incubation of neutrophils with 100 U interferon ml⁻¹ for 10 min at 37 °C resulted in a 2.5-fold increase in *N*-formylmethionyl-leucyl-phenylalanine-stimulated reactive oxygen metabolite generation (as assayed by luminol-dependent chemiluminescence). Pre-treatment of neutrophils with interferon also potentiated their ability to kill *Staphylococcus aureus*, and thus it is proposed that this lymphokine may also enhance neutrophil function *in vivo* under certain pathological conditions.

INTRODUCTION

Polymorphonuclear leucocytes (neutrophils) are phagocytic cells of the immune system which provide the first line of defence in the protection of the host against microbial pathogens. In order to fulfil this role, neutrophils possess a battery of cytotoxic enzymes and associated pathways which are activated or released into the phagocytic vesicle formed after the ingestion of micro-organisms by phagocytosis (Klebanoff & Clark, 1978; Karnovsky & Bolis, 1982). A key event in this killing process is the generation of a series of reactive oxidants during a respiratory burst of non-mitochondrial O₂ uptake (Babior, 1978, 1984; Rossi, 1986), although some pathogens can be killed by bactericidal components whose activity is independent of the requirement for molecular O₂ (Elsbach & Weiss, 1983; Spitznagel, 1984; Vel *et al.*, 1984; Thore *et al.*, 1985).

Whilst neutrophils will respond *in vitro* to a wide variety of chemical and particulate stimuli, considerable interest has focused recently on designing experimental systems which closely simulate their behaviour during acute inflammation. For example, during an inflammatory response neutrophils must be signalled to leave the bloodstream, migrate to the inflamed/infected site and then phagocytose pathogens. During this process, neutrophils are exposed to gradients of a wide variety of inflammatory mediators which may alter their responsiveness prior to their arrival at the inflammatory site. Neutrophils can be 'primed' *in vitro* by low concentrations (usually 10-fold lower than those necessary for activation *per se*) of inflammatory mediators so that their activity upon subsequent exposure to such agents is considerably enhanced (McCall *et al.*, 1979; Van Epps & Garcia, 1980; English *et al.*, 1981; Bender *et al.*, 1983; McPhail *et al.*, 1984; Dewar & Baggiolini, 1985).

Interferon- γ (immune interferon, INF- γ), which is produced during antigenic or mitogenic stimulation of T lymphocytes (Friedman, 1981; Smalley & Borden, 1986), can also modulate the responsiveness of mature, bloodstream neutrophils (Shalaby *et al.*, 1985; Hockland & Berg, 1981; Basham *et al.*, 1984). Some wide-ranging effects have been described, including enhanced antibody-dependent or -independent cell-mediated cytotoxicity (Steinbeck *et al.*, 1986; Perussia *et al.*, 1987) and increased potential to generate reactive oxidants (Berton *et al.*, 1986). Since we have shown that the killing of the bacterium *Staphylococcus aureus* by human neutrophils is

Abbreviations: FMLP, *N*-formylmethionyl-leucyl-phenylalanine; INF- γ , interferon- γ .

crucially dependent upon the generation of reactive oxidants (Edwards *et al.*, 1987), the aim of the present study was to determine whether the bactericidal activity of neutrophils towards this organism was enhanced by exposure to INF- γ .

METHODS

Isolation and purification of neutrophils. Neutrophils were prepared from heparinized venous blood from healthy volunteers, either by a combined dextran/Ficoll sedimentation procedure (Edwards & Swan, 1986), or by centrifugation through M-PRM (Flow Laboratories), as described previously (Edwards *et al.*, 1987). After purification they were suspended in Krebs/HEPES buffer, containing (mM): NaCl, 120; KCl, 4.8; KH_2PO_4 , 4.8; CaCl_2 , 1.3; MgSO_4 , 1.2; HEPES, 25 (pH 7.4); and 0.1% bovine serum albumin.

Growth and opsonization of bacteria. *Staphylococcus aureus* was grown overnight on nutrient agar plates at 37 °C. Cells were then scraped from the plates, suspended in sterile phosphate-buffered saline (PBS, containing 0.15 M-NaCl, 10 mM- KH_2PO_4 , pH 7.4) and the number of viable cells was estimated by measurement of OD_{550} , using suitable calibration curves. Opsonization using pooled serum from healthy donors (stored in portions at -20 °C) was achieved by incubating bacteria ($5 \times 10^8 \text{ ml}^{-1}$) with 10% serum (v/v, final concn) for 30 min at 37 °C (Turner *et al.*, 1986), prior to the addition of 3 vols chilled, sterile PBS and whirlmixing for 30 s. After this, the opsonized bacteria were centrifuged at 1000 g for 15 min in an MSE Centaur 2 centrifuge, washed three times in PBS and finally suspended to known concentration.

Bacterial killing. Neutrophils were suspended in Krebs/HEPES buffer to $10^6 \text{ cells ml}^{-1}$ at 37 °C. At time zero, pre-opsonized bacteria were added (final concn $3\text{--}4 \times 10^7 \text{ ml}^{-1}$) and then samples were aseptically removed at frequent time intervals. These samples were diluted 100-fold in sterile double-distilled water, whirlmixed for 5 min to lyse the neutrophils and then diluted in sterile saline. The number of viable bacteria was then estimated by spread-plating suitably diluted samples onto nutrient agar and incubating overnight at 37 °C.

Incubations with interferon. Purified human INF- γ was diluted in sterile PBS and stored in portions (at a concentration of $50 \text{ U } \mu\text{l}^{-1}$) at -20 °C; portions were not re-frozen after thawing. Neutrophils (10^6 ml^{-1}) were incubated with various concentrations of INF- γ at 37 °C for various lengths of time prior to assay. In all experiments, neutrophil suspensions incubated for identical periods in the absence of INF- γ served as controls.

Chemiluminescence measurements. Chemiluminescence was measured in 1 ml samples containing $10 \mu\text{M}$ -luminol using an LKB 1250 luminometer. In all experiments the neutrophil concentration was $10^6 \text{ cells ml}^{-1}$ and the temperature of operation was 37 °C.

Chemicals. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), purified human INF- γ , HEPES and N-formylmethionyl-leucyl-phenylalanine (FMLP) were from Sigma. All other chemicals were of the highest purity available.

RESULTS

Effect of INF- γ on FMLP-stimulated chemiluminescence

In order to determine whether pre-treatment of neutrophils with INF- γ enhanced either their ability to generate reactive oxidants or their bactericidal activity towards *S. aureus*, it was first necessary to establish the conditions required for maximal potentiation of neutrophil responsiveness. This was achieved by pre-incubating neutrophils with INF- γ and determining the effect on FMLP-stimulated reactive oxidant generation as assayed by luminol-dependent chemiluminescence (Edwards, 1987). Neutrophils incubated with INF- γ at concentrations up to 50 U ml^{-1} for 10 min prior to the addition of FMLP exhibited only a small increase in chemiluminescence response compared to control suspensions incubated under identical conditions in the absence of INF- γ (Fig. 1). However, at concentrations of INF- γ $> 50 \text{ U ml}^{-1}$, a more pronounced effect on FMLP-stimulated chemiluminescence was observed; maximal enhancement (2.5-fold increase) occurred at 100 U ml^{-1} .

The addition of INF- γ at any concentration tested did not stimulate neutrophil chemiluminescence *per se* and a pre-incubation time of $> 2 \text{ min}$ was necessary before any effect on FMLP-stimulated chemiluminescence was observed (Fig. 2). However, as the pre-incubation time with $100 \text{ U INF-}\gamma \text{ ml}^{-1}$ was increased, so the potentiation of the chemiluminescence response increased; the maximal effect was observed when FMLP was added 10 min after the addition of INF- γ . Representative chemiluminescence traces showing the enhancement obtained by pre-incubation of neutrophils with $100 \text{ U INF-}\gamma \text{ ml}^{-1}$ for 10 min prior to the addition of FMLP are shown in Fig. 3.

Fig. 1

Fig. 2

Fig. 3

Fig. 1. Effect of INF- γ on FMLP-stimulated chemiluminescence. Neutrophils (10^6 ml⁻¹, total volume 1 ml) were incubated at 37 °C for 10 min in buffer containing 10 μ M-luminol and varying concentrations of INF- γ . The tubes were then transferred to the luminometer, 1 μ M-FMLP was added (final concn) and the chemiluminescence response was recorded. Data presented have been corrected for the chemiluminescence response obtained in control suspensions incubated under identical conditions in the absence of INF- γ prior to stimulation by FMLP. A 100% increase in chemiluminescence represents a doubling in response. Data presented are typical of those obtained in four separate experiments.

Fig. 2. Time course of INF- γ enhancement of chemiluminescence. Neutrophil suspensions (10^6 ml⁻¹, total volume 1 ml) were incubated at 37 °C in buffer containing 10 μ M-luminol in the presence or absence of 100 U INF- γ ml⁻¹. After incubation for various times, the tubes were transferred to the luminometer and the chemiluminescence response was measured after the addition of 1 μ M-FMLP (final concn). Data presented are corrected for the chemiluminescence responses obtained in FMLP-stimulated suspensions which were incubated for the equivalent times in the absence of INF- γ . Similar results were obtained in three other experiments.

Fig. 3. Typical chemiluminescence traces for the experiments described in the legends to Figs 1 and 2 after the addition of 1 μ M-FMLP (final concn) to neutrophil suspensions which had been incubated for 10 min at 37 °C in the presence (A) or absence (B) of 100 U INF- γ ml⁻¹.

Effect of INF- γ on killing of *S. aureus*

Having established that pre-incubation of neutrophils with 100 U INF- γ ml⁻¹ for 10 min resulted in maximal potentiation of FMLP-stimulated chemiluminescence, it was then necessary to determine whether this treatment also enhanced their bactericidal activity towards *S. aureus*. Neutrophils were incubated for 10 min in the presence or absence of 100 U INF- γ ml⁻¹ prior to the addition of pre-opsonized bacteria. The incubation mixtures were then sampled at timed intervals and viable bacteria estimated. In the absence of INF- γ , the numbers

Fig. 4

Fig. 4. Effect of INF- γ on the killing of *S. aureus* by neutrophils. Neutrophil suspensions (10^6 ml^{-1} , total volume 1 ml) were incubated for 10 min at 37°C in the presence (○) or absence (●) of $100 \text{ U INF-}\gamma \text{ ml}^{-1}$. After this incubation period, 3×10^7 pre-opsonized *S. aureus* were added to each tube and at timed intervals samples were removed for the estimation of viable bacteria, as described in Methods. Data presented are mean values, $\pm$ standard deviations, of at least seven separate experiments.

Fig. 5

Fig. 5. Effect of INF- γ on chemiluminescence during phagocytosis of *S. aureus*. Experimental details as described in the legend to Fig. 4 except that suspensions contained $10 \mu\text{M}$ -luminol. After the addition of bacteria, tubes were transferred to the luminometer and the chemiluminescence traces recorded. Neutrophil suspensions were pre-incubated for 10 min at 37°C in the presence (A) or absence (B) of $100 \text{ U INF-}\gamma \text{ ml}^{-1}$. Similar chemiluminescence traces were obtained in five separate experiments.

of viable bacteria in the suspension rapidly decreased, and 1 h after addition only 30% of the original inoculum remained viable (Fig. 4). However, when neutrophils were pre-incubated with INF- γ prior to the addition of bacteria, their bactericidal activity was potentiated so that the bacteria were killed much more rapidly than in control suspensions. For example, in control suspensions 30% ($\text{SD} \pm 10\%$, $n = 7$) of the bacteria were killed by 30 min whereas with INF- γ -treated neutrophils 70% ($\pm 8\%$, $n = 8$) had been killed by this time; after 60 min incubation there was little difference in the numbers of viable bacteria remaining in control and INF- γ -treated suspensions. Representative traces showing the chemiluminescence responses obtained during phagocytosis of *S. aureus* by control or INF- γ -treated neutrophils are shown in Fig. 5: the chemiluminescence response of INF- γ -treated cells was about twice that of control suspensions.

DISCUSSION

The data presented here clearly demonstrate that pre-treatment of mature, bloodstream neutrophils with $100 \text{ U INF-}\gamma \text{ ml}^{-1}$ for 10 min greatly increases their ability to generate reactive oxidants induced either by exposure to the chemotactic peptide FMLP or during phagocytosis of pre-opsonized *S. aureus*. Furthermore, pre-treatment of neutrophils with this lymphokine also potentiated their ability to kill these bacteria, with over twice as many killed within 30 min after phagocytosis as compared with control suspensions (Fig. 4). This increase in bactericidal activity represented an increase in the initial rate of killing, since the final numbers of bacteria killed were the same in INF- γ -treated and control suspensions. We have shown that killing of *S. aureus* by human neutrophils is crucially dependent upon the generation of oxidants (Edwards *et*

al., 1987), and the results presented here are consistent with this: thus conditions which enhance the ability of neutrophils to generate oxidants also enhance their ability to kill *S. aureus*.

This ability of INF- γ to enhance neutrophil responsiveness appears similar to the 'priming' effects which have been described with other inflammatory mediators. However, in contrast to agents such as FMLP which can 'prime' or 'activate' neutrophils depending on the concentration used, we could not demonstrate 'activation' *per se* by INF- γ at any concentration employed in the present study.

Whereas previous studies have shown that INF- γ can exert a number of effects on mature neutrophil function, none have described those reported here. For example, Berton *et al.* (1986) showed that incubation of neutrophils with recombinant INF- γ at concentrations of 20–50 U ml⁻¹ for 2–4 h was necessary for potentiation of superoxide generation, and Perussia *et al.* (1987) have shown that this lymphokine can protect against the deterioration of neutrophil function which normally accompanies incubation at 37 °C. Recombinant bovine INF- γ was able to enhance both antibody-dependent and -independent cell-mediated cytotoxicity after 2.5 h incubation with bovine neutrophils, but no effect of the ability to ingest *S. aureus* or to generate superoxide was observed (Steinbeck *et al.*, 1986). We show here, for the first time, that pre-incubation of human neutrophils with INF- γ for periods as short as 10 min potentiates their bactericidal activity towards *S. aureus*. Furthermore, luminol-dependent chemiluminescence is similarly enhanced by pre-incubation of neutrophils with this lymphokine for 2–10 min. Since we have shown that efficient killing of *S. aureus* by human neutrophils requires activation of reactive oxidant generation (Edwards *et al.*, 1987), we propose that the enhancement of killing induced by INF- γ resides in the increased ability of neutrophils to generate these oxidants. Further work is necessary to determine the molecular mechanisms underlying this increased bactericidal activity and whether this process involves the NADPH oxidase and/or myeloperoxidase.

Understanding the mechanisms involved during the modulation of the immune response by lymphokines and cytokines is essential if we are to exploit the potential therapeutic advantage offered by these naturally occurring compounds. The observation that INF- γ potentiates the rate of killing of *S. aureus*, and of *Blastomyces dermatitidis* (Morrison *et al.*, 1987), by neutrophils *in vitro* suggests that this lymphokine may be of use clinically in the therapy of infections mediated by these organisms.

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