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In vitro activation of mouse neutrophils by recombinant human interferon-gamma: Increased phagocytosis and release of reactive oxygen species and pro-inflammatory cytokines

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ABSTRACT

Here, we investigated the ability of IFN-γ to modulate the functions of mouse neutrophils in vitro. Neutrophils incubated in the presence of IFN-γ showed enhanced phagocytosis in response to zymosan, opsonized zymosan or precipitated immune complexes of IgG and ovalbumin. The effect of IFN-γ was dose-dependent with an initial response at 10 U/ml and a maximal response at 150 U/ml; 2 h of incubation were required to reach the optimal response level. These stimuli can also induce IFN-γ-pretreated neutrophils to release reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide and hypochlorous acid, as well as granule lysosomal enzymes and the pro-inflammatory cytokines TNF-α and IL-6. We found that increased expression of FcγR, dectin-1 and complement receptors (CRs) correlated with these effects in these cells. The enhancing effect of IFN-γ on the respiratory burst was found to be associated with up-regulation of the $gp91^{phox}$ and $p47^{phox}$ subunits of NADPH oxidase, as measured by their mRNA levels. The enhancing effect of IFN-γ on phagocytosis and ROS release may not only be relevant for the efficient killing of invading microorganisms, but may also produce oxidative stress on adjacent cells, resulting in a possible inflammatory role that could also be favored by the liberation of the pro-inflammatory cytokines TNF-α and IL-6.

1. Introduction

Neutrophils are the most abundant nucleated cells in the blood and constitute the first line of defense protecting the host against microbial pathogens. The antimicrobial function of phagocytes partially depends on the generation of superoxide anion (O_2^-) and other highly reactive oxidants derived during the respiratory burst [1]. Formation of O_2^- is catalyzed by a membrane-associated enzyme system, NADPH oxidase, that is dormant in resting cells and becomes activated during phagocytosis or upon interaction of the cells with suitable soluble stimuli [2–4].

Production of O_2^- in response to a stimulant is potentiated by prior treatment of the phagocytes with activating or priming agents, such as interferon-γ (IFN-γ) [3,5]; lipopolysaccharide (LPS); chemotactic molecules, such as formylated peptides; and cytokines, such as granulocyte-macrophage colony-stimulating factor [6]. IFN-γ has recently been produced by recombinant DNA techniques, and its role as a lymphokine participating in immunological responses is being investigated extensively [7–10]. This recombinant IFN-γ provided a new opportunity to characterize the immunomodulatory properties of IFN-γ, because it is free from other lymphokines, including macrophage-activating factor [11–13]. However, the effects of recombinant IFN-γ on neutrophil function are only recently becoming appreciated [14–18]. Cruz et al. [19] suggested that excessive release of IL-17 during repeated mycobacterial exposure leads to neutrophil recruitment and neutrophil-mediated tissue damage in mice. This response appears to be attenuated by IFN-gamma production during a normal antimycobacterial immune response [20], indicating that neutrophil accumulation and the resulting tissue damage may be caused by the failure of the immune system to contain the infectious agents. Schurgers et al. [21] have highlighted the role of IFN-γ in pathogenesis of human Rheumatoid Arthritis (RA) and collagen-induced arthritis (CIA) in mice.

Previous work in human neutrophils showed that a few hours of IFN-γ treatment induces an enhancement of respiratory burst capability that is dependent on mRNA and protein synthesis; furthermore, IFN-γ-treated neutrophils exhibited no alterations in the amount of cytochrome b_{558} or enhanced expression of receptors [22]. In contrast, Steinbeck et al. [23] were unable to show an effect of recombinant bovine IFN-γ on the respiratory burst of bovine neutrophils. Lieser et al. [24] showed that IFN-γ up-regulates human neutrophil oxidative responses to N-formyl-methionyl-leucyl-phenylalanine (FMLP), but not to phorbol myristate acetate (PMA).

Despite the large number of studies on the regulatory activity of IFN-γ on neutrophil functions [25,26], some conflicting results have been reported regarding phagocytosis and the production of oxygen reactive species when using different stimuli [27–29].

In view of the physiological and pathophysiological importance of the regulatory activity of recombinant IFN-γ on neutrophil functions

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and the diverse, sometimes conflicting results reported, we decided to investigate the effects of this human recombinant cytokine on phagocytosis, the production of reactive oxygen species and the release of lysosome enzymes mediated by different types of immune receptors in mouse neutrophils.

2. Experimental procedure

2.1. Chemicals

Superoxide dismutase (SOD), ferricytrochrome C, Percoll, zymosan, horseradish peroxidase (HRP), bovine albumin serum (BSA), sodium pyruvate, penicillin, streptomycin sulfate, RPMI-1640 tissue culture medium, fluorescein isothiocyanate (FITC) and PKH26 Red Fluorescent Cell Linker were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum was acquired from Life technologies (New York, NY, USA), sodium bicarbonate and ethanol from Merck (Darmstadt, Germany) and the gelatin (microbiological grade) was acquired from Difco Laboratories (Detroit, MN, USA). Phosphate buffered saline (PBS) containing 0.9% NaCl and 0.007 M phosphate buffer, pH 7.2 was used. All the other chemicals and solvents used in this work were of analytical grade and purchased from commercial sources.

2.2. Animals

Male 6- to 8-week-old BALB/c mice were obtained from Ribeirão Preto Medical School, University of São Paulo animal center and maintained under a 12 h light:dark cycle with food and water available ad libitum. The mice were sacrificed by decapitation, and blood was collected in heparin tubes. The animal protocol utilized was approved by the ethics committee of the Faculty of Medicine of Ribeirão-USP (Protocol No. 053/2009).

2.3. Preparation of mouse neutrophils

Neutrophil isolation was performed as described by Boxio et al. [30]. Mouse blood was collected in heparin tubes and added to HBSS-EDTA (without calcium, with magnesium, phenol red and sodium bicarbonate, pH 7.2, 15 mM EDTA, 1% bovine serum albumin (BSA)). After centrifugation (400 *g*, 10 min, 4 °C), cells were resuspended in 1 ml HBSS-EDTA. Cells were then layered onto a three-layer Percoll gradient of 78%, 69% and 52% Percoll diluted in HBSS (100% Percoll = nine parts Percoll and one part 10× HBSS) and centrifuged at 1500 *g* for 30 min at room temperature. The density of each Percoll layer was determined according to the manufacturer's instructions (52%, $\delta = 1.083$ g/ml; 69%, $\delta = 1.090$ g/ml; 78%, $\delta = 1.110$ g/ml). Neutrophils were harvested from the 69/78% interface and the upper part of the 78% layer into 1% BSA-coated tubes after careful removal of cells from the upper phases. After one wash with 2 ml HBSS-EDTA + 1% BSA, the remaining red blood cells were eliminated by lysis with 0.83% (w/v) NH_4Cl at pH 7.2 for 5 min. After a final wash with 2 ml HEPES buffer, the cells were suspended in 1 ml of HBSS and used within 4 h.

2.4. Priming neutrophils

Mouse neutrophils were activated with 150 U/ml recombinant human IFN-γ, produced in *Escherichia coli* (Sigma, St Louis MO, USA) for 2 h prior to all experiments.

2.5. Phagocytosis assay

Immune complexes (ICs) were produced by staining goat red blood cells with (GRBCs) PKH26 according to the manufacturer's instructions and incubating them for 30 min at 37 °C with mouse anti-GRBC antibody. The antibody was produced and purified as previously described by Mantovani [31] and incubated at 37 °C for 15 min with complement (IC-C) to promote opsonization. Zymosan (Zy) was resuspended in carbonate buffer with 25 μg/ml FITC for 30 min at 37 °C and incubated with complement (Opzy). Neutrophils (2×10^6 cells/ml) were incubated at 37 °C for 45 min with 500 μl RPMI-1640 (Sigma St Louis MO, USA) medium containing 10% fetal bovine serum and different phagocytic stimuli, including an immune complex of IgG bound to red blood cell-PKH26 (4×10^6), an immune complex of IgG bound to red blood cell-PKH26 opsonized with complement, Zy-FITC (50 μg), or Zy-FITC opsonized with complement (Opzy-50 μg). The neutrophils were incubated with 150 U/ml IFN-γ for 2 h, and the cells were washed with PBS after incubation. Red blood cells from the immune complex bound to neutrophils were lysed by hypotonic shock as described by Mantovani [31]. The fluorescence of internalized particles was measured by flow cytometry (FACSCanto, BD Biosciences) after fluorescence quenching with trypan blue (2 μg/ml) of the Zy-FITC particles bound to the surface of the neutrophils. The results were analyzed using FlowJo® (Tree Star) software and represented as the mean fluorescence intensity (MFI) per neutrophil.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Cytokines in culture supernatants were measured by a sandwich ELISA using DuoSet (R&D Systems, Minneapolis, MN, USA—TNF-α, DY410 and IL-6, DY406); the procedure was carried out according to the manufacturer's instructions. The tetramethylbenzidine (TMB) reagent set (BD Biosciences) was used as the horseradish peroxidase (HRP) substrate, and absorbance was measured at 450 nm.

2.7. Liberation of granules assay

The β-glucuronidase assay was performed according to methods described by Fishman et al. [32]. Neutrophils (2×10^6) were rested or pre-treated with IFN-γ- (150 U/ml for 2 h) and suspended in RPMI-1640 medium incubated for 1 h at 37 °C with 500 mM cytochalasin B. The supernatant was collected after incubation and centrifuged at 730 *g* for 10 min at 4 °C. This supernatant was kept on ice for subsequent enzymatic assays. The assays used were β-glucuronidase and lactate dehydrogenase. For the β-glucuronidase assay, culture supernatants were incubated with sodium acetate buffer (0.12 M; pH 4.5) and 100 μl of phenolphthalein glucuronate (714 mM). This mixture was incubated at 39 °C for 17 h, and glycine buffer (0.48 M; pH 10.4) was subsequently added. This reaction produces a red colored compound that was measured by a spectrophotometer at 540 nm. The alkaline phosphatase assay was performed as described by Linhardt et al. [33]; for this test, culture supernatants were incubated with 0.25 M Ampol solution (2-amino-2-methyl-1-propanol), 100 mM p-nitrophenylphosphate and 1 M magnesium chloride, pH 10. This mixture was incubated at 37 °C for 30 min with shaking. The reaction was stopped by adding 0.8 N NaOH and was measured by a spectrophotometer at 410 nm.

2.8. Release of superoxide by neutrophils

Extracellular O_2^- release by neutrophils was measured using the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome c assay as previously described by Johnston et al. [34]. Neutrophils, either rested or pretreated with IFN-γ (150 U/ml at 2 h), were suspended in Hanks' containing 1% gelatin to prevent the adhesion of neutrophils to the tubes. A mixture of 800 mM ferricytochrome c and Hanks 15 mM HEPES medium, with or without SOD (15 mg/ml), were incubated with 2×10^6 cells/ml for 5 min at 37 °C. An immune complex of IgG and OVA was prepared by incubating 1 mg/ml OVA for 1 h at 37 °C with anti-OVA antibody, which was prepared and purified as described by Lucisano and Mantovani [35]. Zy or Zy opsonized with complement (Opzy) was prepared as described for the phagocytosis

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