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Suppression by Human Recombinant Gamma Interferon of In Vitro Macrophage Nonopsonic and Opsonic Phagocytosis and Killing

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Although gamma interferon (IFN-γ) exerts profound effects on the state of activation of macrophages, its influence on receptor-mediated phagocytosis and killing of extracellular bacteria is poorly understood. Human monocytes cultured in the presence of human recombinant IFN-γ exhibited an enhanced capacity to produce superoxide anion. Although these cells bound greater numbers of particles via Fc receptors, their capacity to phagocytose by these receptors or to bind or ingest particles via receptors for C3bi, mannose, or unopsonized *Pseudomonas aeruginosa* was substantially depressed in a dose-dependent fashion (0.1 to 1,000 U of IFN-γ per ml). Macrophage phagocytosis of *P. aeruginosa* and *Staphylococcus aureus* opsonized with whole serum or with serum deficient in immunoglobulin or complement was also depressed. Macrophages cultured in the presence of IFN-γ had a diminished capacity to kill both unopsonized and opsonized *P. aeruginosa*. We conclude that IFN-γ inhibits macrophage nonopsonic and opsonic receptor-mediated phagocytosis and killing but enhances oxidative radical generation; its production may exacerbate host tissue damage during chronic infection with extracellular pathogens.

Gamma interferon (IFN-γ) is the principal cytokine for activation of human macrophages (18). As such, it enhances the capacity of macrophages to kill some intracellular pathogens (16). IFN-γ has recently been shown to enhance superoxide anion production and the bactericidal capacity of neutrophils from patients with chronic granulomatous disease (CGD) (7, 8, 21), but its mechanism of action remains to be determined fully.

Although it is clear that IFN-γ exerts profound effects on the oxidative and cidal activities of human monocytic cells, very little is known about its influence on receptor-mediated phagocytosis. There appears to be diversity of effects: selective Fc receptor expression is enhanced (10, 19), mannose receptor expression is down-regulated (15), and complement receptor expression is unaffected (28). Furthermore, certain receptors appear to function poorly despite expression at normal or increased levels (12, 13, 28).

To better understand the full influence of IFN-γ on human phagocytic cells, we investigated its effects on expression and function of the principal phagocytic receptors on monocyte-derived macrophages. The capacity of macrophages to ingest opsonized and unopsonized particles was severely impaired despite enhanced capacity to generate superoxide anion. The observations from these studies form the basis of this report.

MATERIALS AND METHODS

IFN. Human recombinant IFN-γ was kindly provided by Genentech Corp. (South San Francisco, Calif.). IFN-γ was stored in aliquots at −70°C and used within 1 month of thawing.

Particles for phagocytosis. *Pseudomonas aeruginosa* P-1 NM is a nonmucoid revertant of a mucoid strain from a patient with cystic fibrosis (22). It has a rough lipopolysaccharide and is susceptible to the bactericidal effect of normal human serum. *P. aeruginosa* M-2 was a gift from Robert Hancock (Department of Microbiology, University of British Columbia), has a smooth lipopolysaccharide, and is serum resistant. *Staphylococcus aureus* 502A was a gift from Paul Quie (Department of Pediatrics, University of Minnesota). Bacteria were stored frozen at −70°C and were grown fresh overnight on Mueller-Hinton agar for each experiment. Colonies of *P. aeruginosa* were removed with a cotton swab, washed, and resuspended in 10 mM Tris hydrochloride (pH 7.4) (Tris). The *S. aureus* was prepared in a similar manner but was resuspended in phosphate-buffered saline, pH 7.4 (PBS). Erythrocytes opsonized with immunoglobulin G (EIgG), erythrocytes opsonized with C3bi (EC3bi), and zymosan particles were prepared exactly as described previously (24, 29). These particles were used as probes for Fc, C3bi, and mannosyl/fucosyl receptors, respectively (24, 29).

Phagocytic cells. Human monocyte-derived macrophages were prepared exactly as described previously (23). Mononuclear cells from venous blood of healthy adults were cultured (10^6/ml) in Teflon beakers (Savillex Corp., Minnitonka, Minn.) at 37°C in 5% CO_2 for 4 to 6 days, after which the monocytes had acquired the characteristics of macrophages. Cells were washed free of medium immediately prior to each experiment.

Experiments were performed using a relatively pure preparation of monocyte-derived macrophages, prepared by a modification of the method of Wright and Silverstein (29). Percoll (Pharmacia) was first brought to isotonicity by diluting it 1:10 in Hanks balanced salt solution (HBSS) (GIBCO). Twenty-two milliliters of this solution, 1 ml of autologous serum, and 14.7 ml of HBSS were distributed to each of two 40-ml Oak Ridge-type centrifuge tubes (Nalge Co., Rochester, N.Y.) and centrifuged at 30,000 × *g* for 15 min at 4°C. Mononuclear cells (2×10^7 to 5×10^7/ml; maximum, 5 ml) were layered onto the Percoll gradient and centrifuged at 1,000 × *g* for 20 min at 4°C. The upper cell band was aspirated and washed twice. The cells were cultured in Teflon beakers as described above. Monocyte viability was determined by trypan blue exclusion and always exceeded 95%. This method yielded mononuclear cells of which 90 to

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95% were monocytes as determined by morphology on cytocentrifuge preparations and by nonspecific esterase staining.

Cultivation of leukocytes in the presence of IFN-γ. IFN-γ was added to monocyte cultures on the day they were established (day zero) at doses ranging from 0.1 to 1,000 U/ml of medium. Assuming degradation of active IFN-γ during cultivation (28), 66% of the original dose was added on days 2 and 4. Macrophages were washed free of IFN-γ immediately prior to each experiment.

Preparation of opsonins. Pooled normal human serum was prepared from venous blood that was allowed to clot at room temperature for 30 min. Serum was pooled from five normal adults and stored in aliquots at −70°C for no more than 6 months. Under these conditions, the serum retained normal hemolytic complement activity and was bactericidal for *P. aeruginosa* P-1 NM. Hemolytic complement activity was depleted by heating serum at 56°C for 30 min. Serum depleted of antibacterial immunoglobulin was prepared as follows. Venous blood was obtained from a 15-year-old male with X-linked hypogammaglobulinemia, and serum was obtained as described above. *S. aureus* 502A and *P. aeruginosa* M-2 were each grown in 100 ml of tryptic soy broth and were pelleted by centrifugation. To each pellet was added 1 ml of serum. The bacteria were resuspended, and the mixture was incubated on ice for 1 h. After centrifugation, the serum was filter sterilized and stored at −70°C until used.

Polyclonal serum to *P. aeruginosa* P-1 was prepared as follows. Bacteria were grown overnight in L broth without agitation, sedimented by centrifugation, and resuspended in 10% Formalin–normal saline. After 30 min, the bacteria were centrifuged and then washed twice in PBS (pH 7.4). New Zealand White rabbits were injected intramuscularly with the bacteria mixed in an equal volume of complete Freund adjuvant. The rabbits were immunized intramuscularly again on days 18 and 27 with bacteria in incomplete Freund adjuvant and intravenously on day 120 with bacteria in PBS. After an antibody titer exceeding 1:1,000 was demonstrated by enzyme-linked immunosorbent assay and indirect immunofluorescence, the rabbits were exsanguinated by cardiac puncture. Serum was heat inactivated for 30 min at 56°C and stored at −20°C.

Bacteria for phagocytosis experiments were preopsonized by tumbling in 10% serum for 20 min at 37°C, washed in PBS, and adjusted to 10^9 CFU/ml spectrophotometrically. Unopsonized controls were tumbled in PBS.

Assessment of phagocytosis. Ingestion and/or binding of *P. aeruginosa* P-1 NM and M-2, *S. aureus* 502A, EIgG, EC3bi, and unopsonized zymosan was assessed by visual inspection of macrophages plated on glass coverslips as described previously (25). For each condition, the number of particles associated with each of 60 macrophages was quantitated, and the mean was calculated. No attempt was made to distinguish between binding and ingestion for the bacteria or for zymosan. Under these conditions, the majority of EC3bi particles are bound to the leukocyte membrane, so hypotonic lysis of bound erythrocytes was not performed. In the case of EIgG, binding and ingestion of particles was distinguished by washing some coverslips briefly three times in distilled water after all nonattached particles were washed away with PBS. This lysed only the attached EIgG particles and did not affect those which were ingested.

Each phagocytosis experiment was performed on multiple days with macrophages from different donors. Results are expressed as the mean and standard error for the indicated number of experiments.

Assessment of superoxide anion production. Superoxide dismutase-inhibitable reduction of ferricytochrome *c* (27) was measured. A total of 4×10^5 to 1×10^6 macrophages were suspended in HBSS (GIBCO) with 0.1% gelatin (GHBSS). To the cells were added 0.1 mM ferricytochrome *c* (Sigma type IV), 500 U of catalase (Sigma), and 100 ng of phorbol myristate acetate (PMA) (Sigma) to a volume of 1 ml in GHBSS. Negative controls also containing 0.1 mg of superoxide dismutase (Sigma) were run in parallel. Reaction mixtures were incubated for 20 min at 37°C in a shaking water bath, transferred to ice, and centrifuged at 296 × *g* for 10 min at 4°C. Supernatants were promptly removed to iced tubes, diluted one in four with GHBSS, and read on a Spectronic scanning spectrophotometer (Bausch & Lomb) from 560 to 530 nm. Superoxide anion production was calculated from the change in optical density using an extinction coefficient for cytochrome *c* of 21.1 mM^{-1} (26).

Assessment of macrophage-mediated killing of *P. aeruginosa*. Killing of both unopsonized and opsonized *P. aeruginosa* P-1 was assessed by a modification of a method described previously (22). Macrophages cultivated with or without IFN-γ were harvested on day 6 as described above for assessment of phagocytosis. Bacteria were grown overnight in L broth without agitation and adjusted spectrophotometrically to 10^9/ml in PBS (pH 7.4). To 2-ml cryovials (Nalgene) were added 5×10^5 macrophages and 5×10^7 bacteria in a final volume of 250 μl. In some experiments, 1% polyclonal rabbit serum was added. Controls without macrophages or without bacteria (sterility) were always run in parallel. The phagocytosis mixture was rotated end-over-end at 37°C, and samples were removed and diluted in ice-cold distilled water with 0.1% gelatin at 0, 30, and 120 min. Bacteria were further diluted serially and spread on Mueller-Hinton plates for enumeration of viable bacteria.

Statistics. Differences were analyzed by a one-tailed paired Student's *t* test using the NCSS statistical software package (Kaysville, Utah). A *P* value of <0.05 was considered to represent a statistically significant difference.

RESULTS

Superoxide anion production by macrophages cultured in the presence of IFN-γ. Macrophages incubated in the presence of IFN-γ were viable as evidenced by their capacity to exclude trypan blue dye and produce superoxide anion after incubation with soluble or particulate stimuli (see below). IFN-γ-treated macrophages appeared to be smaller than control cells. Whether this was due to a difference in cell volume or spreading characteristics was not determined. Superoxide anion production by normal monocyte-derived macrophages was feeble after stimulation with PMA, but it was enhanced in a dose-dependent fashion when monocytes were cultured in the presence of IFN-γ over a range of 0.1 to 1,000 U/ml (data not shown). This confirmed previous reports and established the appropriate concentrations for use in the phagocytosis experiments.

Phagocytic receptor function of macrophages cultured in the presence of IFN-γ. Phagocytosis or binding of *P. aeruginosa*, opsonized (Fig. 1A) or unopsonized (Fig. 1B), EIgG (Fig. 1C), zymosan (Fig. 1D), and EC3bi (Fig. 1E) was assessed with monocyte-derived macrophages between days 4 and 6 of in vitro maturation. Each experiment was performed on six different days with IFN-γ concentrations from 0.1 to 1,000 U/ml. Results are expressed as the mean and standard error of the means from all experiments. Although there was a wide degree of day-to-day variability with macrophages

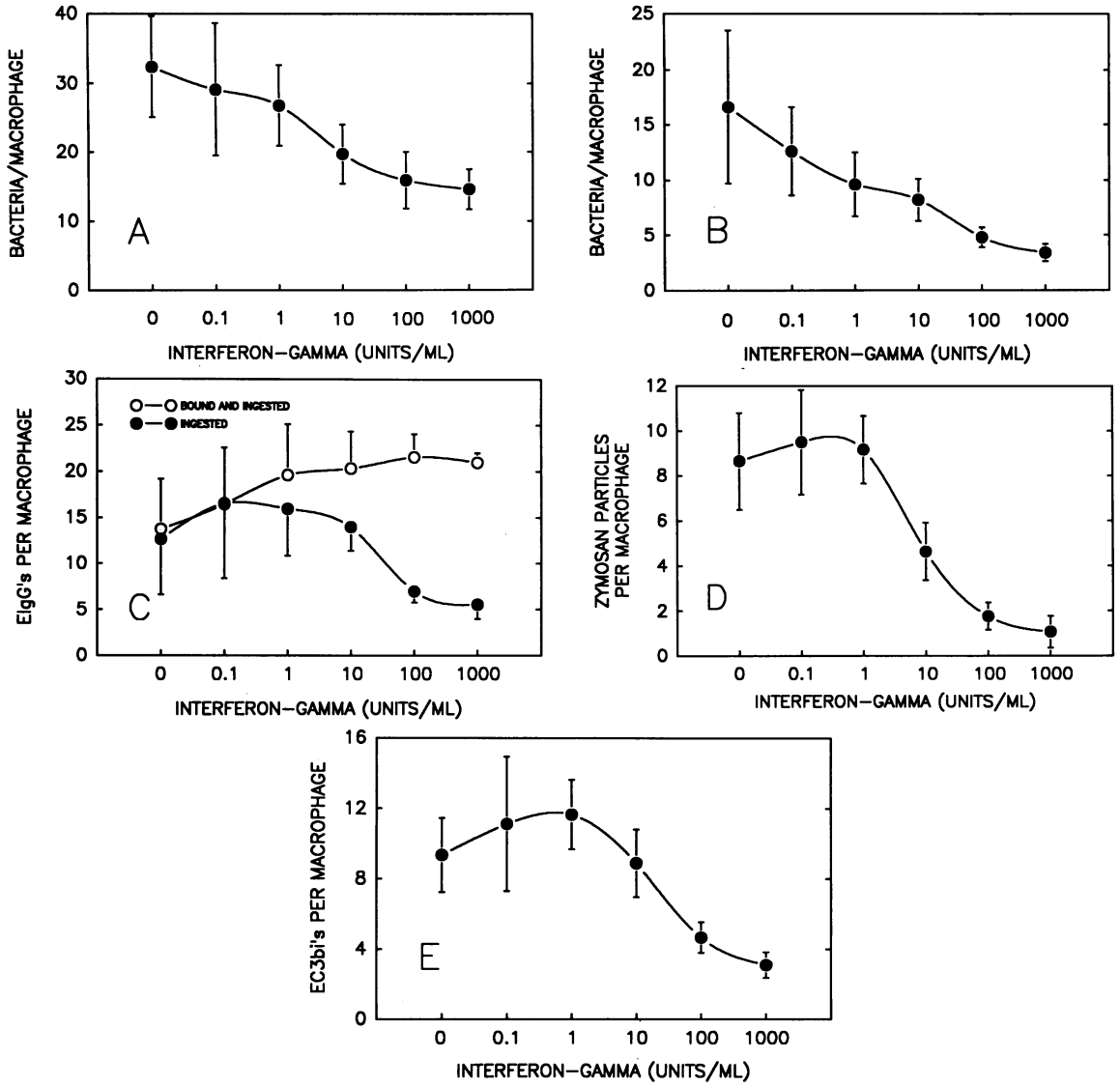

FIG. 1. Effect of different concentrations of IFN-γ on binding and/or ingestion by macrophages of opsonized *P. aeruginosa* P-1 (A), unopsonized *P. aeruginosa* M-2 (B), erythrocytes opsonized with IgG (C), unopsonized zymosan (D), and erythrocytes opsonized with C3bi (E). Mean ± standard error from six experiments. Macrophages were used between days 4 and 6 of culture.

from different donors, a clear dose-response inhibition was demonstrated for each of the particles. The large day-to-day variability in absolute numbers of particles phagocytosed made statistical analysis difficult when data from all experiments were combined. Nonetheless, IFN-γ treatment resulted in significant inhibition of phagocytosis of opsonized and unopsonized *P. aeruginosa* (Fig. 1A and B) and unopsonized zymosan (Fig. 1D) and binding of ECbi (Fig. 1E) ($P < 0.05$ for each particle).

Phagocytosis or binding of unopsonized *P. aeruginosa* P-1 NM was inhibited by IFN-γ over the entire dose range (Fig. 1A). We previously demonstrated that this particle is recognized by macrophage receptors with characteristics of those for both mannose and C3bi (25). Phagocytosis of opsonized *P. aeruginosa* M-2 was inhibited in a similar fashion (Fig. 1B). This particle was opsonized with normal human serum, permitting cooperation of Fc, complement, and nonopsonic receptors.

Effect of IFN-γ on phagocytosis of bacteria opsonized with different sera. Bacteria were next opsonized with sera depleted of specific components to further explore the effects of IFN-γ on specific receptor-mediated phagocytosis. Macrophages were cultivated in the presence of 100 U of IFN-γ per ml, and phagocytosis of *P. aeruginosa* and *S. aureus* opsonized with pooled normal human serum, heated serum (depleted of complement), and adsorbed hypogammaglobulinemic serum (depleted of immunoglobulin) was assessed (Fig. 2). For each condition and in all experiments, a trend was apparent in which phagocytosis by IFN-γ-treated macrophages was depressed compared with that by the untreated control cells. Phagocytosis of *S. aureus* opsonized with adsorbed serum and of unopsonized *P. aeruginosa* was significantly impaired by IFN-γ treatment of the macrophages ($P < 0.05$ for each particle). Statistically significant differences were not attained under the other opsonization conditions. Since it was not possible to differentiate between

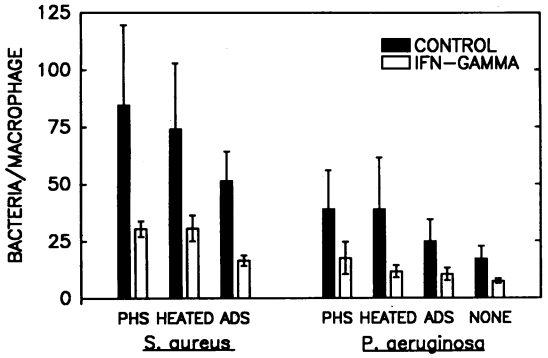

FIG. 2. Effect of IFN-γ (100 U/ml) on ingestion and binding of *S. aureus* 502A and *P. aeruginosa* M-2. The bacteria were preincubated with pooled normal human serum (PHS), serum heated to 56°C for 30 min (heated), adsorbed agammaglobulinemic serum (ADS), or PBS (none). Mean ± standard error from four experiments. Macrophages were used between days 4 and 6 of culture.

macrophage binding and ingestion of bacteria, the competing effects of IFN-γ on these two stages of Fc receptor-mediated phagocytosis (Fig. 1C) may have obscured inhibition of internalization.

Effect of IFN-γ on macrophage superoxide production after incubation with different stimuli. The oxidative burst of IFN-γ-treated macrophages was further assessed by using conventional stimuli (opsonized zymosan and PMA) as well as some of the particles used in the phagocytosis experiments (Fig. 3). Despite the inhibition of phagocytosis of unopsonized *P. aeruginosa* and zymosan and opsonized *P. aeruginosa* and *S. aureus* by cultivation of macrophages in 100 U of IFN-γ per ml, superoxide anion production was not impaired by any of the stimuli tested. Superoxide production was enhanced by IFN-γ treatment when either PMA or opsonized zymosan was used as the stimulus ($P < 0.05$ for each stimulus).

Effect of IFN-γ on macrophage-mediated killing of *P. aeruginosa*. Macrophages cultured in the presence of IFN-γ had a diminished capacity to kill both unopsonized (Fig. 4A) and opsonized (Fig. 4B) *P. aeruginosa* P-1. At 30 min there was no difference between IFN-γ-treated and control macrophages with respect to killing of either unopsonized or opsonized *P. aeruginosa* ($P > 0.05$). However, at 120 min, significantly fewer bacteria were killed by IFN-γ-treated macrophages than by control macrophages, both in the absence (Fig. 4A) and in the presence (Fig. 4B) of opsonization (unopsonized, IFN-γ = 0.128 log increase, control = 0.062 log decrease; opsonized, IFN-γ = 0.119 log increase, control = 0.291 log decrease; $P < 0.05$ for each condition). At both 30 and 120 min, the numbers of unopsonized or opsonized bacteria recovered from the controls lacking macrophages always exceeded those in the presence of macrophages ($P < 0.05$ for each of the four analyses).

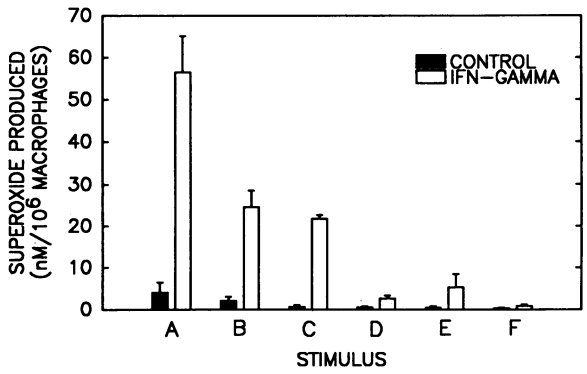

FIG. 3. Effect of IFN-γ (100 U/ml) on superoxide anion production by macrophages incubated with different stimuli. A, PMA (100 ng/10^6 macrophages); B, opsonized zymosan; C, unopsonized zymosan; D, opsonized *S. aureus*; E, opsonized *P. aeruginosa*; and F, unopsonized *P. aeruginosa*. Results are the means ± standard errors from three experiments. Macrophages were used between days 4 and 6 of culture.

DISCUSSION

The observations in this report confirm and expand upon those from previous investigations. We demonstrated that recombinant human IFN-γ has a global suppressive effect on multiple macrophage receptor-mediated functions despite its capacity to up-regulate certain types of macrophage surface molecules, including class II antigens (2, 3) and Fc receptors (10, 19). This up-regulation is associated with an enhanced capacity to mount a respiratory burst. Despite enhancement of both metabolic activity and Fc receptor binding, phagocytic function of both opsonic and nonopsonic receptors was suppressed and capacity to kill unopsonized and opsonized bacteria was compromised. Other investigators have shown previously that the capacity of C3b and C3bi receptors to bind and of Fc receptors to ingest erythrocytes opsonized with the appropriate ligands is inhibited when macrophages are cultivated in the presence of IFN-γ (12, 13, 28). We demonstrated analogous inhibition of both the receptor(s) that recognize unopsonized *P. aeruginosa* and the mannosyl/fucosyl receptor that mediates phagocytosis of unopsonized zymosan. Abrogation of mannose receptor phagocytic function is predicted by previous studies demonstrating suppressed degradation of mannosylated bovine serum albumin by macrophages cultured in the presence of IFN-γ (15). We further demonstrated that IFN-γ interferes with the capacity of macrophages to ingest bacteria opsonized with whole serum, as well as with serum devoid of complement or IgG.

We also demonstrated that macrophages cultured in the presence of IFN-γ had a compromised capacity to kill (or inhibit the growth of) both opsonized and unopsonized *P. aeruginosa*. This was probably due to the depressed capacity to phagocytose the bacteria. Our observations differ from those of other investigators (6, 14) who demonstrated enhanced capacity of IFN-γ-treated neutrophils, monocytes, and pulmonary alveolar macrophages to kill *P. aeruginosa*, *S. aureus*, or *Listeria monocytogenes*. This disparity may be due to inherent differences in those phagocytic cells from the monocyte-derived macrophages used in our investigations.

The therapeutic potential for recombinant human IFN-γ has been demonstrated recently for several different infectious illnesses. In each circumstance, the disease is characterized by an inability of the host to kill intracellular organisms. In the case of lepromatous leprosy (17), intralesional injections of IFN-γ resulted in local improvement. Patients with visceral leishmaniasis who were refractory to therapy with conventional antiparasitic agents improved with the addition of IFN-γ (1). Finally, patients with CGD, who have an impaired capacity to kill ingested microbes due to an inherited defect in oxidative radical-generating capacity (20), demonstrate enhanced oxidative metabolism and partial correction of their bactericidal defect upon receiving paren-

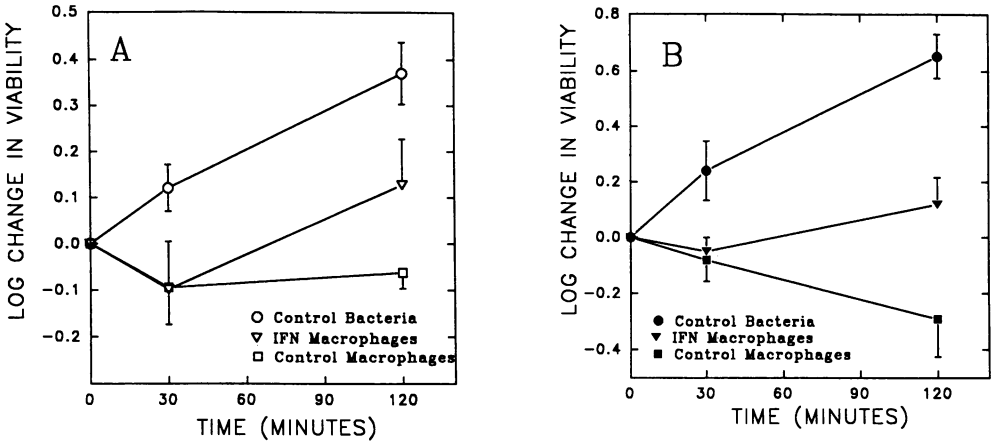

FIG. 4. Effect of IFN-γ (100 U/ml) on macrophage-mediated killing of unopsonized *P. aeruginosa* (A) and of *P. aeruginosa* opsonized with 1% heat-inactivated polyclonal rabbit serum (B). Results represent the means and standard errors from seven experiments performed on separate days. IFN-γ-treated macrophages killed significantly fewer unopsonized and opsonized bacteria than did the control macrophages after 120 min of incubation ($P < 0.05$ for each of the two comparisons). Macrophages were used between days 4 and 6 of culture.

teral therapy with IFN-γ (7). In these patients, IFN-γ partially corrects the defect in superoxide production of both macrophages and neutrophils both in vitro and in vivo (7, 8, 21) and substantially decreases infectious morbidity (11).

The salutary effects of IFN-γ appear to hold for macrophage killing of a diverse range of intracellular parasites, both obligate and facultative (16). This phenomenon may derive largely from an enhanced capacity of phagocytic cells to generate oxidative radicals. However, this may not be the sole explanation, as some patients with CGD improve after receiving IFN-γ therapy despite an incapacity to produce any measurable superoxide anion (11, 21a). In these patients, IFN-γ may enhance some other aspect of oxidative or nonoxidative bactericidal activity. For instance, IFN-γ may promote monocyte killing of *Legionella pneumophila* by a mechanism independent of effects on oxidative radical generation; by interfering with endocytosis of transferrin, it appears to deprive this intracellular pathogen of iron, which is essential for its growth (4). The recent development of an in vitro model of CGD using somatic cell variants of a murine macrophage line may help to unravel the complex effects of IFN-γ on the cidal activity of CGD macrophages (9).

Infection with *Mycobacterium tuberculosis* appears to be facilitated by IFN-γ therapy (5). Although an explanation for this phenomenon has not been found yet, it is clear that eradication of all intracellular pathogens may not be facilitated by IFN-γ therapy.

Our observations may be most relevant to infections with extracellular pathogens at sites where macrophages play a critical role in host defense. Under conditions of subacute or chronic inflammation, IFN-γ may be generated which would be expected to enhance cidal activities of macrophages but depress the capacity of phagocytic receptors to ingest opsonized or unopsonized particles. Such a dual effect may aid the host when infected with obligate intracellular parasites that must gain access to the intracellular environment to survive; ingested organisms would be killed more efficiently, and those which are uningested would be denied the niche they need for survival. For extracellular pathogens, this same inhibition of endocytosis would be expected to work to the host's detriment. Uningested bacteria would survive and replicate in the extracellular milieu, and macrophages would be stimulated by these organisms to produce enhanced amounts of oxidative radicals. The combined effects of pathogen and host products would cooperate in amplifying tissue destruction. Although IFN-γ is an agent with tremendous therapeutic potential, much more must be learned about its mode of action and various in vivo effects before its use can be advocated for the general treatment of infection with intracellular pathogens.

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