Recombinant human γ -interferon induces human monocyte polykaryon formation

(macrophage/osteoclast/hydrogen peroxide/plasminogen activator/macrophage fusion factor)

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ABSTRACT Monocyte or macrophage polykaryons (MP) are seen in different tissues in various inflammatory states and in normal bone (osteoclasts). The factors controlling the formation and the function of MP are not completely understood. This study was designed to evaluate the effects of the lymphokine γ -interferon (IFN- γ) on human monocyte function *in vitro*. Purified recombinant IFN- γ [20–200 units/ml (0.1–1.0 nM)] caused the appearance of MP in cultures of normal human monocytes cultured in 10% unheated autologous serum. The MP were noted by as early as 36 hr of culture with fusion indices of 40%–60% and up to 160 nuclei per cell. The effect was seen with both recombinant IFN- γ and natural IFN- γ produced by Staphylococcal enterotoxin A-stimulated lymphocytes, but IFN- α (leukocyte-derived and recombinant) and IFN- β did not induce MP formation. The activity of the IFN- γ was destroyed by heating at 56°C for 4 hr, incubating at pH 2 for 3 hr, or incubating with antibody against IFN- γ . Populations of monocytes incubated 3 days with 100 units of IFN- γ per ml (0.5 nM) had enhanced capacity to produce H_2O_2 in response to phorbol 12-myristate 13-acetate and increased content of acid phosphatase and plasminogen activator. As determined by autoradiography, the MP did not incorporate [3H]dThd into their nuclei. Thus, the IFN- γ appears to induce MP formation by a process of monocyte fusion, and to “activate” monocytes, as judged by various parameters.

Multinucleated giant cells of mononuclear phagocyte origin [monocyte or macrophage polykaryons (MP)] are seen in various pathologic states including infection, foreign body reactions, cancer, and other conditions of unknown causes such as sarcoidosis and rheumatoid arthritis (1, 2). Osteoclasts are MP thought to be important in the physiologic resorption of bone (3). The factors involved in the formation of MP are not completely understood, and the function of MP seen in various pathologic and physiologic settings is not known.

Lymphokines derived from nonspecific mitogen- or specific antigen-stimulated lymphocytes have been noted to influence the function of mononuclear phagocytes. Factors that induce MP formation have been identified in lymphocyte supernatants (4–8). γ -Interferon (IFN- γ) has been noted to cause “activation” of mouse peritoneal macrophages and cultured human blood monocytes (9–13). The purpose of this study was to determine the effects of purified human IFN- γ on human monocyte function. Results show that the recombinant human IFN- γ from *Escherichia coli* induces MP formation by a process of monocyte fusion and that the treated cells have increased levels of acid phosphatase and plasminogen activator and increased ability to secrete H_2O_2 in response to phorbol 12-myristate 13-acetate (PMA).

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium (no. 430-1600, GIBCO) was formulated with endotoxin-free water and supplemented with 100 units of penicillin per ml/100 μ g of streptomycin per ml/20 mM Hepes/1 mg of dextrose per ml, and was sterilized by filtration (14). The 96-chamber microtiter plates (MicroTest II, no. 3040) were from Falcon; 8-chamber Lab-Tek slides were from Lab-Tek (Naperville, IL); Ficoll-sodium diatrizoate (specific gravity, 1.077; Histo-paque-1077), type II horseradish peroxidase, and *p*-nitrophenyl phosphate disodium were from Sigma. Polyvinylpyrrolidone-coated silica (Percoll) was from Pharmacia. [3H]dThd (2 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear. D-Val-L-Leu-L-Lys-*p*-nitroanilide dihydrochloride (S-2251) was from Helena Laboratories (Beaumont, TX). Urokinase (Calbiochem reference standard) was from Calbiochem-Behring. Affinity-purified plasminogen was a gift from Salvatore Pizzo (Duke University, Durham, NC).

IFN Preparations. Highly purified recombinant DNA-derived, human IFN- γ from *E. coli* (specific activity, 10–50 $\times$ 10⁶ antiviral units per mg of protein) (15) and mouse monoclonal anti-human IFN- γ antibody were supplied by C. Sevastopoulos (Genentech, San Francisco). IFN- γ , partially purified from supernatants of Staphylococcal enterotoxin A-stimulated human lymphocytes (specific activity, 0.7 $\times$ 10⁶ antiviral units per mg of protein), was from Immuno Modulators Laboratories (Houston, TX). Partially purified human α -interferon (IFN- α) (Wellferon) from the lymphoblastoid cell line Namalva was from Burrough's Wellcome (Research Triangle Park, NC). Recombinant IFN- α (clones A and D) were from Hoffmann-La Roche and recombinant IFN- α (α_2) was from Schering. Fibroblast IFN (IFN- β) was from HEM Research (Rockville, MD). For some experiments, the IFN- γ was treated at 56°C for 4 hr, or the pH was adjusted to 2 for 3 hr by adding 1 M HCl with subsequent neutralization by adding 1 M NaOH.

Monocytes. Blood monocytes from normal male and female donors were isolated as described by using a combination of Ficoll-sodium diatrizoate and Percoll density-gradient sedimentation (16). The donors gave written informed consent according to a protocol approved by the Duke University Clinical Investigation Committee. Cells isolated by this technique were composed of 80%–95% monocytes with the remainder being lymphocytes. In general, there were <4–6 platelets per monocyte. The monocytes (3×10^5 per microtiter well) were incubated for 1 hr at 37°C, and then the nonadherent cells were removed by two washes with saline. This process resulted in monolayers with >95% monocytes. In selected experiments, monocytes were isolated by adherence to serum-coated plastic (17); although the yield was

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Abbreviations: IFN- γ , γ -interferon; IFN- α , α -interferon; IFN- β , β -interferon; MP, monocyte polykaryon; PMA, phorbol 12-myristate 13-acetate.

lower using this procedure, the cells were 100% monocytes. All cultures were in 10% autologous unheated serum.

H₂O₂, Acid Phosphatase, and Plasminogen Activator Assays. H₂O₂ generation in response to 200 nM PMA was measured with monolayers of monocytes in microtiter plates as described by using phenol red as an oxidizable substrate in the presence of horseradish peroxidase and glucose (18). Acid phosphatase activity in monocyte lysates (0.05% Triton X-100) was measured using *p*-nitrophenyl phosphate disodium as substrate (19). Plasminogen activator activity in monocyte lysates was done using the chromogenic substrate S-2251, in the presence and absence of plasminogen (20); only the plasminogen-dependent activity is reported. A Multiskan automatic eight-channel photometer (Flow Laboratories) was used to measure the absorbances of the phenol red (600 nm) and the *p*-nitrophenol (405 nm) in microtiter plates. Protein was measured by the method of Lowry *et al.* (21).

Autoradiography. Monocyte monolayers were established from 1.0×10^6 monocytes per chamber on eight-chambered Lab-Tek slides with chambers of 1-cm² surface area (14). The cells were incubated over the 3 day culture period with or without 0.5 nM IFN- γ with [³H]dThd at 5 μ Ci/ml in each chamber. The mouse fibrosarcoma cell line 3T12 was used for rapidly proliferating control cells. The slides were fixed and processed as described (14).

Cell Staining. Cells adherent to the plastic in the microtiter chambers were stained with either Wright's stain, nonspecific esterase (22), or acid phosphatase with or without tartrate (23). MP are defined as monocytes having more than two nuclei per cell. Cells were counted on the stained microtiter plates using a $\times 20$ objective and a $\times 10$ eyepiece. The fusion index was calculated with the following formula: fusion index = (number of nuclei in MP in a given area per total number of nuclei in that same area) $\times 100$.

RESULTS

The control cells, after 3 days of culture in 10% autologous unheated serum, were generally mononuclear and had the appearance of normal blood monocytes. However, the cells cultured in 100 units of IFN- γ per ml (or 0.5 nM) were multinucleated (Fig. 1). In these MP, the nuclei were usually positioned in the center of the cells, and many looked like the Langhans giant cells that are seen in patients with granulomatous diseases. The MP measured from 40 to 2000 μ m in diameter. The cells stained positively for nonspecific esterase and acid phosphatase. The acid phosphatase activity was resistant to inhibition by tartrate. The time course for the formation of the MP in the presence of 100 units of IFN- γ per ml (0.5 nM) as assessed by the fusion index (the percentage of the total number of nuclei that are within MP) is shown in Fig. 2. The MP were noted as early as 36–48 hr, and the peak effect was seen by 72–96 hr. Occasionally, the control cultures had a small amount of fusion (as seen in this figure), while in other experiments, there was none. Fig. 3 shows a dose response for the IFN- γ in cultures stained after 3 days of incubation. The MP formation was manifest as an increase in the fusion index, an increase in the percentage of cells that are polykaryons, and an increase in the mean number of nuclei per polykaryon. The effect appeared to be maximal at doses of 50 and 200 units/ml, with a decrease at doses of 300–400 units/ml. MP with as many as 162 nuclei per cell were noted.

It appears that the IFN- γ acts directly on the monocytes to cause the MP formation. It is conceivable that small numbers of contaminating lymphocytes could be induced to secrete fusion factors by the IFN- γ , but we feel this is very unlikely. The MP formation was similar in cultures of monocytes isolated by the Ficoll-sodium diatrizoate/Percoll method as well as the serum-coated plastic method. This sig-

FIG. 1. Photomicrographs of Wright's-stained monocytes in microtiter chambers. The cells were cultured for 3 days without (A and C) or with (B and D) 100 units of IFN- γ per ml (0.5 nM). (A and B, $\times 20$; C and D, $\times 100$.)

nifies that the presence of small numbers of lymphocytes in the Ficoll-sodium diatrizoate cells did not affect the MP formation. Also, cells from the human promyelocytic leukemia cell line HL-60 form polykaryons after culture with the IFN- γ , indicating clearly that lymphocytes are not required for the reaction (unpublished data). The MP were noted in cells cultured with the recombinant IFN- γ from Genentech and in those cultured with the partially purified IFN- γ from human lymphocytes stimulated with Staphylococcal enterotoxin A (data not shown). However, none of the preparations of

FIG. 2. Time course of monocyte polykaryon formation in cultures without or with 100 units of IFN- γ per ml (0.5 nM). The points show the means $\pm$ SEM of triplicate samples.

FIG. 3. Dose response of IFN- γ in inducing monocyte polykaryon formation as assessed by fusion index (●), percent polykaryons (○), and number of nuclei per cell (Δ). The points show the means $\pm$ SEM of triplicate samples.

IFN- α or IFN- β enhanced MP formation. IFN- α from the lymphoblastoid cell line Namalva, and the recombinant DNA-*E. coli*-derived IFN- α 's from Hoffmann-La Roche (clones A and D) and from Schering (α_2) did cause some rounding up of the monocytes, but no MP formation was seen (Fig. 4). The effect of the IFN- γ was abolished by heating the preparation at 56°C for 4 hr, exposing it to pH 2 for 3 hr, or incubating it with mouse monoclonal antibody against IFN- γ (Fig. 5).

In MP with as many nuclei as these, it would appear most likely that they formed by fusion of cells. Monocytes are generally thought to be nondividing cells, but it was conceivable that the giant cells were formed by nuclear division without cytoplasmic division (endomitosis). We investigated this by incubating the monocytes with the IFN- γ and [3 H]dThd over a 3-day culture period and then doing autoradiography on the cells. As noted in Fig. 6, the multinucleated monocytes showed no grains over their nuclei (indicating no [3 H]dThd incorporation into their DNA), while the control, rapidly proliferating fibrosarcoma cells had heavy labeling over their nuclei. The nuclear labeling index of the MP was 0, while that of the fibrosarcoma cells was 100%.

After the monocytes were cultured for 3 days with 100 units of IFN- γ per ml, certain functional changes were noted

FIG. 4. Comparison of polykaryon formation induced by IFN- γ and various forms of IFN- α or IFN- β . The points show the means $\pm$ SEM of triplicate samples. ●, IFN- γ ; Δ, Wellferon- α ; ▲, Roche- α A; ○, Roche- α D; □, Schering- α_2 ; ▽, IFN- β .

FIG. 5. The effect of heat, low pH, and monoclonal antibody against IFN- γ on IFN- γ -induced monocyte polykaryon formation. The IFN- γ (100 units/ml; 0.5 nM) was heated at 56°C for 4 hr (Heat), kept at pH 2 for 3 hr (Acid), or incubated with 1000 units of anti-IFN- γ antibody per ml (Antibody). Bars represent the means $\pm$ SEM of triplicate samples.

(Fig. 7). The ability of the population of IFN-treated cells to produce H_2O_2 in response to PMA was dramatically increased. Likewise, the content of the acid hydrolase acid phosphatase was increased, as was the content of the neutral protease plasminogen activator.

DISCUSSION

This study demonstrates that highly purified recombinant human IFN- γ and natural IFN- γ derived from *Staphylococcal enterotoxin A*-stimulated lymphocytes induce the formation of MP from uninuclear monocytes by a process of cell fusion, and that populations of monocytes cultured with IFN- γ have increased levels of acid phosphatase and plasminogen activator and increased H_2O_2 production in response to PMA. Several investigators have noted in the past that a "macrophage fusion factor" in lymphokine supernatants would promote polykaryon formation in populations of rabbit alveolar macrophages (4-6), rat alveolar macrophages (7), mouse peritoneal macrophages (6), and human blood monocytes (8). Postlethwaite *et al.* (8) showed that the soluble protein produced by antigen- or mitogen-stimulated human lymphocytes, which induced human monocyte multinucleated giant cell formation, was destroyed by trypsin or heating at 56°C for 45 min. Based on gel filtration studies, the factor had a M_r of $\approx 60,000$. The procedures that others have

FIG. 6. Photomicrographs of autoradiograms of monocyte polykaryons (A) or 3T12 fibrosarcoma cells (B). ($\times 200$.)

FIG. 7. H₂O₂ production in response to 200 nM PMA, acid phosphatase content, and plasminogen activator content of control and IFN- γ -treated monocytes. The cells were incubated 3 days with 20 units of IFN- γ per ml (0.1 nM) or 100 units of IFN- γ per ml (0.5 nM) and then assayed. Bars represent the means $\pm$ SEM of triplicate samples.

used to produce IFN- γ (24, 25) make it likely that the supernatants used by others studying macrophage fusion factors contained IFN- γ . Our results suggest that IFN- γ can certainly serve as a human monocyte fusion factor and that it may be identical to that studied by Postlethwaite *et al.* (8).

Recent investigators have shown that murine IFN- γ activates mouse peritoneal macrophages for tumor-cell killing and serves as a mouse "macrophage activating factor" (9, 10, 26, 27). Nathan *et al.* and Murray and co-workers showed that human IFN- γ enhances the ability of cultured human monocytes to produce H₂O₂ in response to PMA and to kill microbes *in vitro* (11–13). Our data confirm the enhancement of the H₂O₂-producing ability and also show that the IFN- γ increases the monocyte content of acid phosphatase and plasminogen activator. These features have been correlated with activation of mouse macrophages and human monocytes (28–30). Le *et al.* recently reported that IFN- γ activates normal human monocytes for tumor cytotoxicity (31). Our work has also shown that IFN- γ enhances tumor cell killing by normal human monocytes and human peritoneal macrophages (unpublished data).

The MP formed *in vitro* after culture of normal blood monocytes with IFN- γ morphologically resemble the multinucleated giant cells seen *in vivo* in various immunological and nonimmunological reactions in humans (1, 2). We hypothesize that IFN- γ may be involved in granuloma formation *in vivo* and that the administration of IFN- γ could influence the formation of MP *in vivo*. Also, we speculate that IFN- γ may be involved in the transformation of blood monocytes into the multinucleated osteoclasts, the cells involved in resorption of bone in physiologic and pathologic states (3).

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