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Research Article

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Killing of Intracellular *Leishmania donovani* by Lymphokine-stimulated Human Mononuclear Phagocytes

EVIDENCE THAT INTERFERON-γ IS THE ACTIVATING LYMPHOKINE

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ABSTRACT We have found that the crude lymphokines, which prime the human monocyte-derived macrophage to generate H_2O_2 and exert microbicidal activity against intracellular *Leishmania donovani*, are rich in interferon (IFN)-γ (600–3,000 U/ml). To determine the role of this specific lymphocyte product in macrophage activation, lymphokines were pretreated with a monoclonal antibody that neutralizes human IFN-γ. Antibody exposure completely abolished the capacity of both mitogen- and antigen-stimulated lymphokines to either enhance macrophage H_2O_2 release or induce leishmanicidal activity. In addition, partially purified and pure recombinant human IFN-γ were as effective as crude lymphokines in activating macrophages, and 3 d of treatment with 300 U/ml resulted in a seven- to eightfold increase in H_2O_2 generation and the intracellular killing of both *L. donovani* promastigotes and amastigotes. The ability of crude lymphokines to induce monocytes and macrophages from a patient with chronic granulomatous disease to kill *L. donovani* promastigotes was similarly abrogated by anti-IFN-γ antibody, and could also be achieved by IFN-γ alone. These results suggest that IFN-γ is the key macrophage-activating molecule present within human lymphokines, and indicate that IFN-γ can enhance both the oxygen-dependent and -independent antiprotozoal mechanisms of human mononuclear phagocytes.

INTRODUCTION

In a recent report (1), we demonstrated that soluble lymphocyte products (lymphokines) readily stimulate monocyte-derived human macrophages to display microbicidal activity against both the promastigote and amastigote forms of *Leishmania donovani*, the etiologic agent of visceral leishmaniasis. Although the activity towards this intracellular parasite appeared to be primarily H_2O_2-dependent, lymphokines also induced macrophages from patients with chronic granulomatous disease (CGD)[1] to kill ingested promastigotes and inhibit amastigote replication (1). These observations suggested that lymphocyte products can enhance and thus regulate both the oxygen-dependent and -independent microbicidal responses of the human mononuclear phagocyte. The present report extends this analysis by demonstrating that interferon (IFN)-γ appears to be the molecule within human lympho-

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[1] *Abbreviations used in this paper:* CGD, chronic granulomatous disease; Con A, concanavalin A; IFN, interferon; LDA, *L. donovani* amastigotes; LDP, *L. donovani* promastigotes; PMA, phorbol myristate acetate; SEA-IFN-γ, staphylococcal enterotoxin A (SEA)-stimulated partially purified IFN-γ.

kines that activates the macrophage's antiprotozoal mechanisms.

METHODS

Cells. Human peripheral blood mononuclear cells were separated by standard techniques and prepared as described (1). Adherent monocytes were cultivated on glass coverslips at 37°C in 5% CO_2-95% air for 10–23 d before use (1). The culture medium, RPMI 1640 supplemented with 15% heat-inactivated heterologous human serum, penicillin (100 U/ml), and streptomycin (100 μg/ml), was changed every 3 d.

Preparation of lymphokines and IFN-γ. Mitogen-stimulated lymphokines were generated by incubating mononuclear cells (3×10^6/ml) in RPMI 1640 medium containing 15% fresh heterologous human serum, antibiotics, and 15 μg/ml of concanavalin A (Con A) (type III, Sigma Chemical Co., St. Louis, MO) (1). Antigen-induced lymphokines were prepared by cultivating mononuclear cells (3×10^6/ml) from Sabin-Feldman dye-test-positive donors with *Toxoplasma gondii* lysate antigen (50 μg/ml) (1). After 48 h at 37°C in 5% CO_2-95% air, supernatants were centrifuged to remove cells, sterilized by filtration, and stored at 4°C. Control (sham) lymphokines, which did not enhance macrophage oxidative or antileishmanial activity, consisted of (*a*) cultures incubated alone with Con A added at the end of the 48 h period, and (*b*) cells from dye-test-negative donors cultivated with *T. gondii* antigen (1).

IFN-γ was prepared by stimulating peripheral blood leukocytes with 0.02 μg/ml of staphylococcal enterotoxin A (SEA) and purifying the culture supernatant by sequential column chromatography to a specific activity of 1×10^7 antiviral U/mg of protein (2).[2] Genentech, Inc. (S. San Francisco, CA) provided pure recombinant human IFN-γ synthesized by *Escherichia coli* (3). The antiviral activity of both lymphokines and IFN-γ preparations was determined using a cytopathic effect inhibition assay with vesicular stomatitis virus in WISH cells (4). Antiviral activity was measured against a laboratory IFN-γ standard, and is expressed as laboratory units (2).

Antibodies to IFN. Monoclonal IgG_1 anti-human IFN-γ antibody, derived from a GIF-1 hybridoma supernatant, contained 5,000 neutralizing U/ml (2). Sheep antisera to human IFN-α and IFN-β were National Institutes of Health reference preparations G-026-502-568 and G-028-501-568, respectively. For neutralization, dilutions of lymphokine or SEA-stimulated IFN-γ were incubated for 45 min at 37°C with 2 neutralizing U of antibody per measured unit of IFN-γ (2).

Macrophage antileishmanial activity and H_2O_2 release. After >10 d in culture, monocyte-derived macrophages were treated for 1–3 d with (*a*) standard medium alone; (*b*) active or sham lymphokines; (*c*) lymphokines preincubated with anti-IFN antibodies; or (*d*) IFN-γ. The culture medium was not changed during this period. Cells were then washed, and challenged for 1 h with either 5×10^6 *L. donovani* promastigotes (LDP) or amastigotes (LDA) (1). Uningested parasites were removed by washing, and coverslips were reincubated in medium alone. The course of intracellular infection was assessed by enumerating the number of *L. donovani*/100 cells (1). The scopoletin method was used to assay macrophage H_2O_2 release after triggering with 100 ng/ml of phorbol myristate acetate (PMA) (1).

[2] Rubin, B. Y. Manuscript in preparation.

RESULTS

IFN-γ activity of crude lymphokines. Con A was an effective stimulus for IFN-γ generation, and in 22 experiments, culture supernatants of mononuclear cells from seven donors contained 1,877±193 U/ml (mean±SEM; range, 800–3,000 U/ml). After exposure to *T. gondii* antigen, cells from three dye-test-positive individuals also readily secreted IFN-γ, and supernatants contained 1,417±240 U/ml (range, 600–3,000 U/ml, 12 experiments). None of the 10 mitogen or antigen control (sham) lymphokines had detectable IFN-γ (<10 U/ml).

Effect of IFN antibodies on lymphokine activity. In the absence of stimulation, monocyte-derived macrophages display no antileishmanial activity, and support the intracellular replication of both LDP and LDA (1). 3 d of pretreatment with 5–20% lymphokine, however, induces these cells to exert leishmanicidal effects (1). To determine if the IFN-γ present in these supernates participated in macrophage activation, mitogen- and antigen-prepared lymphokines were incubated with a monoclonal antibody to IFN-γ before addition to macrophage cultures. As shown in Fig. 1, this antibody, which does not neutralize either IFN-α or IFN-β (2), abolished the capacity of lymphokines to induce antileishmanial activity. In two experiments, treatment with antisera to human IFN-α and IFN-β had no effect on lymphokine activity, and exposure of

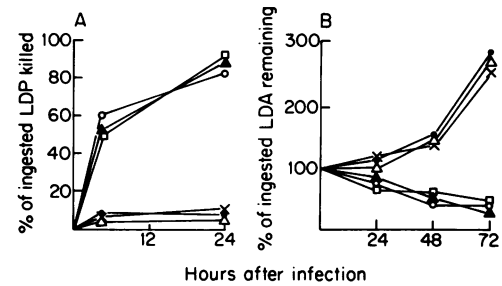

FIGURE 1 Monocytes were cultivated for 10–20 d, and then activated for 3 d before challenge with LDP (*A*) or LDA (*B*) with 10% Con A lymphokine (containing 3,000 U/ml of IFN-γ undiluted (○), IFN-γ antibody-neutralized 10% lymphokine (X), 300 U/ml of SEA-IFN-γ (□), antibody-neutralized SEA-IFN-γ (△), 300 U/ml of recombinant IFN-γ (▲), or medium alone (●). Results are the means of three experiments in which the standard errors were <10% of the means. After the 1-h infection period (time zero), 48–75% of macrophages were infected with LDP or LDA with 72–188 parasites/100 cells. Parasite ingestion by control and treated macrophages was comparable as previously reported (1). The antileishmanial activity induced by 3 d of treatment with 20% *Toxoplasma* antigen-stimulated lymphokine (containing 1,500 U/ml of IFN-γ) was similar to that shown for Con A supernates, and was also abolished by anti-IFN-γ antibody (data not shown).

macrophages to anti-IFN-γ antibody alone for 3 d before infection did not alter the intracellular replication of LDP or LDA (data not shown).

Induction of antileishmanial activity by IFN-γ. Although the preceding results indicated that IFN-γ was required for lymphokines to activate normal macrophages to a leishmanicidal state, these experiments did not address the possibility that IFN-γ achieved its effects by acting in concert with other supernatant products. Thus, we next treated macrophages with two IFN-γ preparations, one partially purified (2) and the other purified to homogeneity (3). The results of these experiments (also shown in Fig. 1) confirmed that IFN-γ alone could effectively activate macrophages in a fashion similar to crude lymphokines. In each case, the activity induced by SEA-IFN-γ was abrogated by pretreatment with anti-IFN-γ monoclonal antibody. Additional experiments, which compared the dose-responses (10–300 U/ml) and duration (1–3 d) of IFN-γ stimulation required for the induction of leishmanicidal activity, indicated a close correlation between the effects achieved by various dilutions of IFN-γ-containing lymphokines and comparable amounts of either IFN-γ preparation (data not shown). Although some antileishmanial activity could be induced by pretreatment for 1–2 d with 50–100 U/ml of IFN-γ, 3 d of exposure to 300 U/ml of IFN-γ or equivalent concentrations of lymphokine (e.g., a 10% dilution of a supernatant containing 3,000 U/ml) stimulated optimal antileishmanial activity (Fig. 1). Similar to our previous results with crude lymphokines (1), the addition of 300 U/ml of SEA- or pure IFN-γ after infection only had no effect on LDP or LDA intracellular replication. In addition, exposing LDP and LDA to either IFN-γ preparation (300 U/ml) or to lymphokine (10–20%) for 1 h at 37°C had no direct effect on parasite viability, as judged by the subsequent survival and replication of treated organisms.

Enhancement of oxygen-dependent and -independent activity by IFN-γ. Our prior studies with normal and CGD monocyte-derived macrophages indicated that crude lymphokines not only stimulate the normal macrophage's capacity to generate H_2O_2 and exert oxygen-dependent antileishmanial activity, but also enhance the effects of an oxygen-independent mechanism as well (1, 5). In experiments performed in parallel with those illustrated in Fig. 1, we also found that the capacity of both mitogen- and antigen-induced lymphokines to augment the normal macrophage's oxidative activity is dependent on IFN-γ, and that this molecule by itself can prime macrophages as effectively as lymphokines to produce H_2O_2 (Table I). These results suggested, then, that enhanced oxidative and antiprotozoal activity, two closely correlated and key markers of lymphokine-induced macrophage activation (1, 5–8), were both IFN-γ dependent.

To determine whether the apparent oxygen-independent antileishmanial activity, which lymphokines also confer upon mononuclear phagocytes (1), was mediated by IFN-γ, monocytes and macrophages from

TABLE I
Macrophage H_2O_2 Generation

Pretreatment*	Experiment (age of cells) I (13 d)	II (14 d)	III (23 d)	Fold increase in H_2O_2 release
Medium (control)	72±8	88±9	23±4	—
Lymphokines				
10–20%	368±22	526±16	152±19	6.2
10–20% + Ab	61±9	97±10	28±6	1.1
IFN-γ (300 U/ml)				
SEA-IFN	381±18	487±14	197±15	6.8
SEA-IFN + Ab	48±6	102±8	16±4	0.8
Recombinant IFN	420±16	ND‡	256±20	8.4

* Monocytes were cultivated for 10, 11, or 20 d and then stimulated for 3 d (as described in the legend to Fig. 1) before triggering with PMA, 100 ng/ml (1). Results are the means±SEM of triplicate coverslips, and are expressed as nanomoles of H_2O_2 per milligram of adherent cell protein per 90 min (1). In experiments I and II, two Con A lymphokines (LK) (each containing 3,000 U/ml of IFN-γ undiluted) were used at a 10% concentration. In experiment III, a *Toxoplasma* antigen-stimulated LK, containing 1,500 U of IFN-γ/ml, was used at a 20% concentration. Ab, anti-IFN-γ monoclonal antibody.
‡ Not done.

TABLE II
Effect of Lymphokines and IFN-γ on LDP Killing by CGD Cells

Cells and pretreatment	Percentage of ingested LDP killed by 2-d monocytes* 4 h	2-d monocytes* 24 h	13-d macrophages‡ 4 h	13-d macrophages‡ 24 h
Normal				
Medium (control)	72	93	8	12
Con A LK (10%)	66	90	52	78
CGD§				
Medium (control)	0	23	2	8
Con A LK (10%)	19	93	13	61
Con A LK + Ab	1	10	3	9
SEA-IFN-γ (300 U/ml)	21	89	15	66
SEA-IFN-γ + Ab	0	6	4	10
Recombinant IFN-γ (300 U/ml)	31	90	19	63

* Freshly plated monocytes were treated from the outset of culture for 2 d with the indicated agents (see Fig. 1 legend) before challenge with LDP. Results are the means of duplicate coverslips at each time point.

‡ Monocytes first cultivated for 10 d were then treated for 3 d before LDP challenge. In both experiments, the Con A lymphokine (LK) used contained 3,000 U/ml of IFN-γ undiluted.

§ 1-d monocytes from this previously studied CGD patient (1) released 31±4 nmol of H_2O_2/mg cell protein per 90 min after PMA triggering, while 1-d normal cells release 1,013±58 nmol/mg protein per 90 min (1). In addition, none of this patient's monocytes or macrophages reduced nitroblue tetrazolium (NBT) in response to PMA or LDP ingestion (1), and there was no increase in H_2O_2 release or NBT reduction after 2 or 3 d of treatment with either 10% lymphokine (LK) or 300 U/ml of SEA- or recombinant IFN-γ.

a previously studied CGD patient (1) were treated before LDP challenge with crude lymphokine, antibody neutralized lymphokine, or IFN-γ alone. The results shown in Table II again demonstrated the activating effects of IFN-γ, and indicated that the oxygen-independent activity induced by lymphokines also appeared to be IFN-γ dependent.

IFN-γ production by cells from a patient with leishmaniasis. It was also pertinent to our studies of host cell-mediated immune responses in leishmaniasis (9) to determine if specific antigen could stimulate cells from a *Leishmania*-infected patient to secrete IFN-γ. The patient examined had extensive *Leishmania mexicana* cutaneous infection (19 separate skin lesions) and was tested three times—before, during, and 6 mo after successful antimony treatment (10). On these three occasions, in response to *L. mexicana* promastigote antigen (50 μg/ml), his cells produced <10, 20, and 600 U/ml of IFN-γ, respectively, and this in vitro secretory activity appeared to correlate with the clinical status of his infection. Mononuclear cells from two healthy controls showed no IFN-γ production (<10 U/ml) upon exposure to *L. mexicana* antigen.

DISCUSSION

It has been well established that soluble lymphocyte products effectively stimulate human monocyte-derived macrophages to display enhanced oxidative metabolic activity (1, 6) and to kill or inhibit the replication of the intracellular protozoa, *T. gondii* (7, 11), *Trypanosoma cruzi* (8), and *L. donovani* (1). However, the activating molecule(s) within the crude lymphokines heretofore used have not been identified. Our observations extend these studies of macrophage activation and its expressions by demonstrating that (*a*) human lymphokines, including those stimulated by specific protozoal antigen, are rich in IFN-γ activity; (*b*) an anti-human IFN-γ monoclonal antibody abolishes the capacity of lymphokines to induce the activated state; and (*c*) that IFN-γ alone can activate human macrophages in a fashion similar to crude lymphokines. Although the latter finding suggests that the IFN-γ in these lymphokines does not require a cofactor to achieve macrophage activation, we have not excluded the possibility that other supernatant factors may act synergistically with IFN-γ, or under different conditions, induce activation by themselves. As judged by the apparent specificity of our anti-IFN-γ antibody (2) and the absence of any neutralizing effect of antisera to IFN-α and IFN-β, however, one can probably exclude a primary macrophage-activating role for IFN-α or -β in the lymphokine preparations we used.

The finding that IFN-γ alone was capable of enhancing oxygen-dependent as well as -independent antileishmanial activity is consistent with our prior results which demonstrated that crude lymphokines (shown here to contain high levels of IFN-γ) successfully stimulate monocytes and macrophages from both normal individuals and patients with CGD to kill *L. donovani* (1). In addition, in a number of previous studies, various IFN preparations have also been reported to enhance the antimicrobial activity of different cell populations against other nonviral intracellular pathogens, including *T. gondii* (12–15), *Rickettsia akari* (16), and *Mycobacterium tuberculosis* (17). In separate studies using the same monoclonal anti-IFN-γ antibody and additional lymphokines and other IFN-γ preparations (including pure, recombinant IFN-γ), we have also recently observed that the capacity of human monocyte-derived macrophages to respond to lymphokine with (*a*) enhanced oxidative burst activity and oxygen-dependent killing of *T. gondii* (18), and (*b*) oxygen-independent inhibition of in-

tracellular *Chlamydia psittaci* and *T. gondii* replication (5, 19) are both dependent upon and can be induced by IFN-γ.

Taken together, these studies suggest that a single molecule secreted by sensitized T lymphocytes (20) can regulate both the oxygen-dependent and -independent antimicrobial mechanisms of human macrophages, and readily activate these effector phagocytes to exert activity against a diverse group of intracellular pathogens. Because of observations such as these, it would seem reasonable to next determine if patients with visceral leishmaniasis, an infection characterized by suppressed lymphocyte activity (21) and persistent parasitization of host tissue macrophages, have a defect in IFN-γ generation, and then explore the possibility of replacement therapy with IFN-γ.

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