INFECTION AND IMMUNITY, May 1990, p. 1296–1300
0019-9567/90/051296-05$02.00/0

Vol. 58, No. 5

In Vivo Activation of Macrophage Oxidative Burst Activity by Cytokines and Amphotericin B

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Received 21 August 1989/Accepted 2 February 1990

Alterations in macrophage oxidative burst activity following in vivo administration of recombinant murine gamma interferon (IFN-γ), recombinant murine tumor necrosis factor alpha, and the antifungal antibiotic amphotericin B were investigated. Mice were given intraperitoneal injections of these agents alone and in combination, and the oxidative responses of their resident peritoneal macrophages to challenge with *Histoplasma capsulatum* or zymosan particles were measured 1 to 5 days later. Various degrees of enhanced oxidative burst activity were achieved following treatment with each agent. However, a synergistic response was observed only when mice were treated with the combination of recombinant murine IFN-γ and amphotericin B. These results not only confirm the dual role of amphotericin as an antifungal agent and as an immunomodulator but also suggest that IFN-γ may serve as a useful adjunct in the treatment of intracellular fungal infections.

The microbicidal activity of macrophages (Mφs) is determined largely by their level of activation. This generally correlates with the ability of phagocytic cells to generate an oxidative burst (25, 28), although nonoxidative mechanisms of microbial destruction have also been recognized (2, 6, 7, 24). Avoidance or inhibition of the oxidative burst by parasites is therefore often necessary, although not always sufficient, for intracellular survival. Gamma interferon (IFN-γ) is believed to be the principal Mφ-activating factor responsible for regulating enhanced toxic oxygen metabolite release (26) and fungistatic (2, 39, 41) and fungicidal mechanisms (1, 5, 13, 33). However, there is increasing evidence to suggest that it may also interact with other known immunomodulators, including the cytokine tumor necrosis factor alpha (TNF-α) (10, 12, 16, 33) and bacterial endotoxin (20, 32, 39), to potentiate these effects. In addition, the modulating effects of the antifungal agent amphotericin B (AmB) on the host immune system have also been demonstrated (21, 32; J. R. Little and J. E. Wolf, Clin. Immunol. Newsl. 8:183–185, 1987).

Previous studies have shown that the intracellular parasite *Histoplasma capsulatum* impairs Mφ oxidative (11, 40) and antimicrobial mechanisms (37, 41). Although Mφs exposed to IFN-γ in vitro acquire the ability to restrict intracellular proliferation of *H. capsulatum*, a second priming signal (cytokine) is necessary in order to overcome *Histoplasma*-mediated inhibition of the oxidative burst (39). In this study, we examined murine Mφ oxidative function, specifically with regard to *H. capsulatum* challenge, following in vivo treatment with cytokines and AmB.

MATERIALS AND METHODS

Animals. Female 8-week-old AKR/J mice were obtained from Jackson Laboratory, Bar Harbor, Maine.

***H. capsulatum* G217B.** Heat-killed lyophilized yeast phase cells of *H. capsulatum* G217B (40) were obtained from G. Kobayashi, Washington University, St. Louis, Mo., and were stored at 4°C until used.

Reagents. Recombinant murine IFN-γ (rMuIFN-γ) (<0.016 endotoxin units per 5.2×10^6 IFN units) and recombinant murine TNF-α (rMuTNF-α) (<0.022 endotoxin units per 3.3×10^7 TNF units) were generously provided by Genentech, Inc. (San Francisco, Calif.). Fungizone, the preparation of AmB used, was generously donated by E. R. Squibb & Sons (Princeton, N.J.); zymosan, *Escherichia coli* lipopolysaccharide, latex spheres, and superoxide dismutase from *Aspergillus niger* were purchased from Sigma Chemical Co. (St. Louis, Mo.); ferricytochrome *c* was obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wis.); Proteose Peptone was purchased from Difco Laboratories (Detroit, Mich.); Hanks buffered saline solution, Dulbecco modified Eagle medium, and phosphate-buffered saline were obtained from GIBCO Laboratories (Grand Island, N.Y.); defined fetal bovine serum and defined donor equine serum were obtained from HyClone Laboratories, Inc. (Logan, Utah) and were heated at 56°C for 1 h before use.

Isolation of phagocytic cells. Normal murine peritoneal exudate cells were induced by intraperitoneal (i.p.) injection of 1.0 ml of 10% Proteose Peptone and were harvested from ascitic fluid 72 h later (9). Erythrocytes were lysed by treatment with Tris-buffered ammonium chloride (22). Elicited peritoneal Mφs were then suspended at 4×10^6 to 5×10^6 nucleated cells per ml of medium, and cells from 1.0-ml samples were allowed to adhere to glass cover slips (13 mm in diameter) (Thermanox; Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) at 37°C for at least 2 h before assaying.

In vivo Mφ priming. AmB-primed peritoneal Mφs were elicited by i.p. injection of 0.5 mg of AmB or 0.25 mg of deoxycholate (as a control) in 1 ml of 5% dextrose solution and were harvested 72 to 96 h later. rMuIFN-γ- or rMuTNF-α-primed peritoneal exudate cells were obtained either 24 or 72 h following a single i.p. injection of cytokine in 1 ml of phosphate-buffered saline or were elicited via a sequential injection schedule in which an initial i.p. injection of 1 ml of 10% Proteose Peptone (72 h prior to harvest) was followed by a second i.p. injection of 10,000 U of rMuIFN-γ or 330 U of rMuTNF-α in 1 ml of phosphate-buffered saline 24 h prior to harvest. In experiments in which AmB and cytokines were used in combination, AmB was injected i.p. 72 to 96 h and rMuTNF-α or rMuIFN-γ was administered (in the same

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TABLE 1. O_2^- production by in vivo-primed Mϕ[a]

Injection schedule[b]	Stimulus		
	Zymosan	*H. capsulatum*	Mϕ alone
Proteose Peptone (−72 h)	7.0 ± 1.2	1.0 ± 0.6	1.0 ± 0.4
rMuIFN-γ (−24 h)	5.5 ± 3.4	2.8 ± 0.3	1.0 ± 0.5
Proteose Peptone (−72 h)-rMuIFN-γ (−24 h)	12.7 ± 3.7[c]	3.7 ± 0.9[c]	2.1 ± 0.9
rMuTNF-α (−24 h)	12.0 ± 3.5[c]	2.0 ± 1.5	1.0 ± 0.6
Proteose Peptone (−72 h)-rMuTNF-α (−24 h)	12.5 ± 3.1[c]	1.5 ± 0.6	0.5 ± 0.1
rMuTNF-α (−72 h)	27.0 ± 2.3[c]	12.0 ± 5.8[c]	1.0 ± 0.8
Deoxycholate (−72 h)	4.2 ± 0.3	0.4 ± 0.4	2.2 ± 0.9
AmB (−72 h)	14.0 ± 5.5[c]	6.3 ± 1.8[c]	2.3 ± 0.7
AmB (−72 h)-rMuIFN-γ (−24 h)	29.0 ± 3.0[c]	15.5 ± 3.5[c]	2.2 ± 0.2

[a] Nanomoles of O_2^- released by cover slip-adherent Mϕs harvested from mice injected according to the above schedules, measured by ferricytochrome *c* reduction as described in Materials and Methods. Results are presented as mean ± standard deviation for three replicate experiments from each group.
[b] Indicates timing of i.p. injections relative to harvest of peritoneal Mϕs.
[c] Statistically significant differences between primed and control Mϕ responses by the Student *t* test; $P < 0.05$.

concentrations as above) 72 or 24 h (respectively) prior to harvest.

Zymosan and *H. capsulatum* preparations for Mϕ triggering. Zymosan and heat-killed lyophilized *H. capsulatum* were suspended Hanks buffered saline solution to yield concentrations of approximately 10^8 yeast cells per ml.

Superoxide anion (O_2^-) release. We used a modification of the ferricytochrome *c* reduction assay described by Johnston (17). Elicited Mϕs (5×10^6/ml) were allowed to adhere to 24-mm wells for 2 h at 37°C with 5% CO_2 in Dulbecco modified Eagle medium with 10% fetal calf serum. Supernatants were aspirated, and the Mϕs were washed once in prewarmed Hanks buffered saline solution (without phenol red) before a subsequent 2-h incubation in 1 ml of Hanks buffered saline solution containing 360 μmol of ferricytochrome *c* with or without 40 μg of superoxide dismutase per ml. O_2^- release was quantified by measuring the absorbance of culture supernatants in a spectrophotometer (Bausch & Lomb Spectronic 1001; Bausch & Lomb, Inc., Rochester, N.Y.). The change in extinction coefficient at 550 nm for ferricytochrome *c* reduction was 21,000 M^{-1} cm^{-1}, which was applied to the difference in absorbance of culture supernatants in the presence or absence of superoxide dismutase. After completion of the ferricytochrome *c* reduction assay, each preparation was Giemsa stained and evaluated microscopically to confirm particle phagocytosis and uniform density of intact adherent Mϕs.

Statistical analysis. Results are presented as means ± standard deviation, as analyzed by the Student *t* test.

RESULTS

Effect of in vivo administration of rMuIFN-γ, rMuTNF-α, and AmB on Mϕ oxidative metabolism. Peritoneal Mϕs from mice treated with a single dose of rMuIFN-γ or rMuTNF-α i.p. were harvested for measurement of superoxide release 24 or 72 h postinjection. These Mϕs were compared with those from other mice who received i.p. injections of Proteose Peptone (72 h prior to harvest) followed by injections of rMuIFN-γ or rMuTNF-α (24 h prior to harvest). Doses of the cytokines used were those previously reported to affect Mϕ function in vivo (4, 18, 21, 36). Superoxide release by these populations of in vivo-primed Mϕs challenged with *H. capsulatum* or zymosan was compared with that of unprimed Proteose Peptone-elicited peritoneal Mϕs. In vivo administration of rMuIFN-γ moderately enhanced zymosan- and *H. capsulatum*-triggered superoxide release if given after a prior injection of Proteose Peptone but not when given alone. In contrast, a marked superoxide response to both triggering agents was observed with Mϕs from mice which had received rMuTNF-α alone 72 h before harvest. Superoxide production after other rMuTNF injection schemes exhibited only moderate enhancement.

Studies have shown that AmB can influence a variety of immune functions in addition to its direct antifungal activity (21, 32; Little and Wolf, Clin. Immunol. Newsl., 1987). To evaluate its effect on Mϕ oxidative activity, Mϕ monolayers from mice which had received an i.p. injection of AmB (Fungizone) 3 to 5 days prior to harvest were stimulated in vitro with *H. capsulatum* or zymosan. Control mice were similarly injected with a 5% glucose solution containing sodium deoxycholate in a concentration which approximates that found in AmB. This injection scheme was derived from preliminary experiments (data not shown) which demonstrated this to be the optimal interval between in vivo treatment and Mϕ harvest. AmB- (but not deoxycholate-) elicited Mϕs exhibited enhanced superoxide release upon challenge with *H. capsulatum* and zymosan (Table 1). In the absence of specific triggers, no oxidative burst activity was observed.

Synergistic effect of in vivo combination therapy. To determine whether individual immunomodulators could interact synergistically to influence Mϕ activation, mice were injected with various combinations of rMuIFN-γ, rMuTNF-α, and AmB and their peritoneal Mϕs were assayed as described above. Unlike studies reported previously (10, 12, 16, 39) in which in vitro exposure of Mϕs to the combination of rMuIFN-γ and rMuTNF-α markedly augmented oxidative activity, in vivo treatment of mice with these agents failed to enhance Mϕ oxidative responses beyond that observed with either agent alone (data not shown). Similarly, there was no benefit to using the combination of AmB and rMuTNF-α. However, when mice were treated with AmB followed by rMuIFN-γ, their Mϕs exhibited synergistic zymosan- and *H. capsulatum*-stimulated oxidative burst activity (Table 1). This appears to correlate with in vitro studies in which AmB has been shown to interact with rMuIFN-γ to produce Mϕ activation for increased tumor cell cytotoxity (21).

Characteristics of peritoneal Mϕs from mice treated with in vivo immunomodulators. The mechanism(s) responsible for the enhanced Mϕ oxidative burst following in vivo treatment of mice with immunomodulators is unknown but may in-

TABLE 2. Phagocytic index of in vivo-primed Mϕs[a]

Mϕ population	% Phagocytosis	Mean no. of particles per Mϕ
Proteose Peptone	82.7 ± 9.6	2.4 ± 1.8
rMuIFN-γ	80.0 ± 8.2	2.9 ± 2.1
rMuTNF-α	70.0 ± 11.5	2.7 ± 2.1
AmB	82.5 ± 5.0	3.3 ± 2.0
rMuTNF-α + AmB	77.5 ± 9.6	3.1 ± 2.1
rMuIFN-γ + AmB	95.0 ± 5.7	6.3 ± 2.9

[a] Giemsa-stained Mϕ monolayers were evaluated microscopically for phagocytosis of zymosan or *H. capsulatum* particles as described in Materials and Methods. Each value represents the mean ± standard deviation of four observations for each group.

volve the recruitment of activated cells into the peritoneal cavity. Conversely, these agents may act via direct activation of the existing local population of Mϕs. In order to try to distinguish between these two possibilities, we quantitated the number of Mϕs harvested per animal by using the above-described injection schedules and the number of Proteose Peptone-elicited cells as a baseline for comparison. None of the Mϕ harvests, regardless of in vivo inducer, varied significantly from an average of 6×10^6 to 1×10^7 cells per mouse, so this parameter could not be used as a quantitative marker for immunopotentiation.

Mϕ monolayers were also evaluated for variation in phagocytic index. No significant differences were observed in the populations obtained from mice which had been injected with Proteose Peptone, rMuIFN-γ, rMuTNF-α, AmB, or rMuTNF-α and AmB in combination. Approximately 80% of the Mϕs in these preparations contained intracellular particles with an average of 2 to 4 particles per cell. In contrast, AmB–rMuIFN-γ-primed Mϕs contained more than twice as many particles per cell, and the proportion of actively phagocytic cells approached 100% in all fields surveyed (Table 2).

DISCUSSION

Expression of Mϕ activation has been defined in terms of enhanced oxidative, tumoricidal, and/or microbicidal activity (25, 28, 30). Among the different environmental signals which may play a role in the activation process, either independently or in concert, are IFN-γ (28), TNF-α (16, 33, 39), bacterial endotoxin (20, 29), granulocyte-monocyte colony stimulating factor (3, 38), other less well-characterized cytokines (3, 27), and the polyene antifungal compound AmB (21, 32; Little and Wolf, Clin. Immunol. Newsl., 1987). IFN-γ has been shown to enhance the antimicrobial activity of Mϕs against a variety of intracellular bacterial and protozoan parasites (23). Depending on in vitro experimental conditions, Mϕs activated with IFN-γ are rendered either fungistatic (2, 39, 41) or fungicidal (1, 13, 16, 33) against the pulmonary dimorphic fungi *Coccidioides immitis*, *Blastomyces dermatitidis*, and *H. capsulatum*. In the host, however, more than one signal (cytokine) may be necessary in order to achieve full Mϕ activation and microbicidal potential. Alternatively, Mϕs may need to combine with other immune cells to kill invading fungi.

Previously, we described inhibition of the oxidative burst in unprimed murine peritoneal Mϕs by the intracellular fungal parasite *H. capsulatum* (40). This inhibition could be overcome if Mϕs were exposed in vitro to a combination of priming agents (rMuIFN-γ and recombinant human TNF-α or lipopolysaccharide) but not to these agents used individually (39). In this study, we investigated the effect of in vivo administration of rMuIFN-γ, rMuTNF-α, and AmB on the oxidative burst, specifically with regard to triggering by *H. capsulatum*. We found that although Mϕs from mice treated with either rMuIFN-γ or rMuTNF-α exhibited enhanced oxidative burst activity following *H. capsulatum* or zymosan challenge (Table 1), the magnitude of the responses differed. In addition, there appeared to be differences in the length of time (between injection and harvest) required for activation. The relative efficacy of priming for enhanced oxidative burst activity among the various in vivo injection schedules suggests that these immunomodulators exert their effects by inducing a set of distinct in vivo conditions. This may be a consequence of differences in the extent of cytokine diffusion, rate of degradation, competition for surface receptors, and effect on cells other than Mϕs. Since each cytokine has a broad range of immunomodulatory activities, including Mϕ priming for enhanced oxygen radical release (28, 39), induction of other cytokines, and mediation of their effects (3, 16, 33), further studies are required to elucidate their complex in vivo interactions. It may be that these agents act on different Mϕ subpopulations with varying capabilities or on the same population of Mϕs via induction of separate pathways of activation. One approach to investigating these alternatives might be to employ monoclonal antibodies to identify Mϕ-monocyte surface antigen markers.

The antifungal agent AmB may act in part against invading fungi by promoting host immune responses (21, 32; Little and Wolf, Clin. Immunol. Newsl., 1987). In vitro (data not shown) and in vivo (Table 1) studies from our laboratory confirm that AmB injected into mice alters the in vitro oxidative capacity of peritoneal Mϕs stimulated with *H. capsulatum* and zymosan particles. We further investigated this potentially dual role of AmB in the treatment of intracellular fungal infections by evaluating its ability to enhance the effects of rMuIFN-γ and rMuTNF-α on Mϕ activation. Mϕs from mice treated with AmB and rMuIFN-γ displayed synergistic oxidative activity when triggered with zymosan and *H. capsulatum* (Table 1). While the mechanism(s) underlying its immunopotentiation is unknown, AmB has been shown to induce secretion of TNF-α by murine Mϕs in vitro (8, 14) and to increase serum levels of colony-stimulating factor in mice given AmB i.p. (19). Moreover, recent evidence suggests that, in addition to its effect on cell membrane sterols (15), AmB may exert antifungal effects by promoting oxidative damage (35).

The contribution of oxidative metabolism to antifungal activity in *H. capsulatum*-parasitized Mϕs is still unknown. Although inhibition of intracellular proliferation of *H. capsulatum* yeasts appears to be mediated in part by mechanisms independent of the oxidative burst, possibly through elaboration of cationic peptides (31, 34) or enhanced phagosomal-lysosomal fusion (2), enhanced oxidative responses of in vivo cytokine-activated Mϕs may provide a critical line of defense during the initial exposure to *H. capsulatum*. Since AmB also influences the acquisition of the activated state as an adjunct to its antifungal activity and produces synergistic oxidative responses when used in combination with IFN-γ, it may help render a fungistatic response fully fungicidal. This may provide a rationale for new immunotherapeutic approaches to the treatment of systemic fungal infections.

ACKNOWLEDGMENT

This work was supported by a research grant from the American Lung Association.

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