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Recombinant and Natural Gamma-Interferon Activation of Macrophages In Vitro: Different Dose Requirements for Induction of Killing Activity against Phagocytizable and Nonphagocytizable Fungi

ELMER BRUMMER,* CHRISTINE J. MORRISON, AND DAVID A. STEVENS

Division of Infectious Diseases, Department of Medicine, Santa Clara Valley Medical Center and Institute for Medical Research, San Jose, California 95128, and Stanford University, Stanford, California 94305

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Recombinant murine gamma-interferon (IFN) and supernatants from concanavalin A (ConA)-stimulated spleen cells were tested for their ability to activate resident peritoneal macrophages (Mϕ) for fungicidal activity. Mϕ monolayers pulsed overnight with IFN exhibited significantly enhanced fungicidal activity against *Candida albicans* (44 ± 12 versus 0.0%) and *Blastomyces dermatitidis* (34 ± 1 versus 3 ± 3%). The effect of IFN was dose dependent; however, less IFN (10 U/ml) was required to activate Mϕ to kill phagocytizable *C. albicans* than to kill nonphagocytizable *B. dermatitidis* (1,000 U/ml). ConA-stimulated spleen cell supernatants were also able to activate Mϕ for fungicidal activity against both fungi. The capacity of ConA-stimulated spleen cell supernatants to activate Mϕ for fungicidal activity was neutralized in the presence of antibody to murine IFN. ConA-treated monolayers acquired the ability to kill *C. albicans*, but not *B. dermatitidis*, which was shown to be associated with residual (10%) lymphocytes in the monolayers. Lipopolysaccharide (0.001 to 10 μg/ml) failed to consistently activate Mϕ for fungicidal activity. These data show that IFN can exert an immunoregulatory role on Mϕ defense against these fungal pathogens.

Activation of macrophages, first described in vivo (4, 22, 29, 43) and then in vitro (2, 14, 18, 32) with lymphokines, has received extensive study and application (48) in the past 10 years. Many reports show lymphokine activation of macrophages for tumoricidal (28, 35, 46, 56), protozoicidal (2, 5, 20, 33, 38), bactericidal (14, 18, 23, 38), rickettsiacidal (10, 35), and fungicidal (3, 55) activities. Recently, an active component in lymphokine-containing supernatants was identified as gamma-interferon (gamma-IFN) (30, 31, 34, 38, 42, 47, 53), and recombinant DNA-produced gamma-IFN was shown to have the same activity (26, 39, 40). However, lymphokines may contain compounds other than gamma-IFN that are capable of activating macrophages (17, 19, 31, 41, 44). Receptors for IFN on macrophages (13) and fibroblasts (1) have been reported, and mechanisms of activation by IFN are under investigation.

Unlike polymorphonuclear neutrophils, which kill many pathogenic fungi such as *Candida albicans*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, and *Sporothrix schenckii*, and under certain conditions *Blastomyces dermatitidis* (7, 9), resident peritoneal macrophages generally lack such activity against pathogenic fungi (3, 8, 24, 25, 45). On the other hand, macrophages activated in vivo (8, 45) or in vitro (3, 55) with lymphokines acquired the ability to kill some fungal pathogens. Tentative identification of the factor(s) active in lymphokines as IFN has been made in the case of macrophage activity against *H. capsulatum* (55). In this study, we used highly purified recombinant DNA-produced murine gamma-IFN and antibody to IFN to demonstrate that IFN activates resident peritoneal macrophages against phagocytizable *C. albicans* and nonphagocytizable *B. dermatitidis*. Furthermore, IFN was identified as an active factor in supernatants from cultures of spleen cells stimulated with concanavalin A (ConA). These findings demonstrate a new and previously undefined role for IFN in macrophage defense against fungal pathogens.

* Corresponding author.

MATERIALS AND METHODS

Animals. Male BALB/cByJIMR mice (8 to 12 weeks of age) were obtained from the breeding colony of the Institute for Medical Research, San Jose, Calif., for use in these experiments.

Reagents and media. Tissue culture medium RPMI 1640 with L-glutamine, Eagle minimum essential medium (MEM), heat-inactivated fetal bovine serum, and penicillin-streptomycin (P/S; 10,000 U/ml and 10,000 μg/ml, respectively) were obtained from GIBCO Laboratories (Grand Island, N.Y.). Complete tissue culture medium (CTCM) consisted of 89 ml of RPMI 1640 medium, 10 ml of fetal bovine serum, and 1 ml of penicillin-streptomycin. ConA and α-methyl-D-mannoside (aMM; grade III) were purchased from Sigma Chemical Co., St. Louis, Mo. Lipopolysaccharide (LPS-W), prepared from *Escherichia coli* O128:B12, was obtained from Difco Laboratories, Detroit, Mich. Preservative-free heparin was purchased from Scientific Products, Sunnyvale, Calif., and 96-well, half-area tissue culture plates were obtained from Costar Co., Cambridge, Mass. Murine gamma-IFN produced by recombinant DNA technology and hyperimmune rabbit antibody (0.155 mg/ml) made against murine gamma-IFN was supplied by Genentech Corp., South San Francisco, Calif. (13, 49). The latter, purified by affinity chromatography (49), neutralized 1.6×10^4 (preparation 1) or 4.5×10^4 (preparation 2) antiviral (IFN refer-

ence) U/ml. The amount of antibody added to IFN in experiments was calculated by these reference points to neutralize the IFN present.

Fungi. A strain of *B. dermatitidis* (ATCC 26199), which is virulent in mice (6), was used in these experiments. Inocula of *B. dermatitidis* were prepared from organisms grown for 48 to 72 h on blood agar plates at 37°C (21). *C. albicans* Sh 27 was grown in yeast nitrogen broth (Difco) at 35°C for 3 to 4 days. The fungi were washed twice in 4 ml of saline, pelleted cells were suspended in CTCM, and units (single or multicellular) were counted with a hemacytometer. Viable CFUs were determined by plating 1 ml of appropriate dilutions in quadruplicate on blood agar plates.

Peritoneal macrophages. Peritoneal cells (PC) were collected from the abdominal cavity of each mouse by repeated lavage with a total of 10 ml of MEM containing heparin (10 U/ml). PC from six mice were pelleted by centrifugation (200 × *g*, 10 min) and pooled. The number of cells per milliliter was determined with a hemacytometer. PC were dispensed (0.1 ml of 5×10^6/ml of CTCM) into half-area Costar plate wells, the plate was incubated at 37°C in 5% CO_2–95% air for 2 h, and then nonadherent cells were aspirated, and each well was washed once with CTCM. When the number of nonadherent cells was subtracted from the number of incubated PC, the average number of adherent cells per well was 2.5×10^5. Adherent cells have been shown previously to be >90% nonspecific esterase positive (8).

Treatment of macrophages. Macrophage monolayers were incubated overnight (37°C, 5% CO_2–95% air) with 0.1 ml of CTCM or CTCM containing 1 to 100,000 U of IFN/ml, 1 ng to 100 μg of ConA per ml, 0.1 ng to 10 μg of LPS per ml, or spleen cell culture supernatants. After incubation, culture supernatants were aspirated, monolayers were challenged with 0.1 ml of *C. albicans* (10,000 CFU/ml of CTCM) or *B. dermatitidis* (5,000 CFU/ml of CTCM), and then 0.01 ml of fresh mouse serum was added. Cocultures were incubated at 37°C in 5% CO_2–95% air for 2 or 4 h. Cultures were harvested by aspiration and repeated washing of culture wells with distilled water. Microscopic examination of washed wells indicated that there was complete removal of macrophages. Each culture and well washings were contained in a final volume of 10 ml. To determine the number of CFU per culture, 1 ml of the 10 ml of distilled water dilution was plated on a blood agar plate, and colonies were counted after 2 days (*C. albicans*) or 5 days (*B. dermatitidis*) of incubation at 37°C.

Spleen cell supernatants. Spleen cells were obtained from normal mice and cultured as described previously (7). Supernatants were generated by incubation of 10^7 spleen cells in 2 ml of CTCM with or without 5 μg of ConA per ml in 24-well tissue culture plates (3047; BD Labware, Oxnard, Calif.) at 37°C in 5% CO_2–95% air for 1 to 3 days. Supernatants were harvested from pooled cultures by centrifugation (400 × *g*, 10 min) and filtration through 0.45-μm pore-size filters (Millipore Corp., Bedford, Mass.). Portions of supernatants were stored at −70°C until use.

Pretreatment of monolayers. Peritoneal cell monolayers were treated with 0.1 ml of anti-T-cell serum (Accurate Chemical Co., Westbury, N.Y.) diluted 1:10 in MEM at room temperature for 1 h. This was followed by aspiration of liquid from the wells and addition of Low-Tox M rabbit complement (Accurate Chemical Co.) diluted 1:10 in MEM (0.1 ml per well). Controls consisted of monolayers treated with anti-T-cell serum–MEM, MEM–Low-Tox M complement, or MEM–MEM. After incubation at 37°C for 1 h, liquid was removed from the wells, and the monolayers were treated overnight with CTCM or CTCM containing ConA (5 μg/ml).

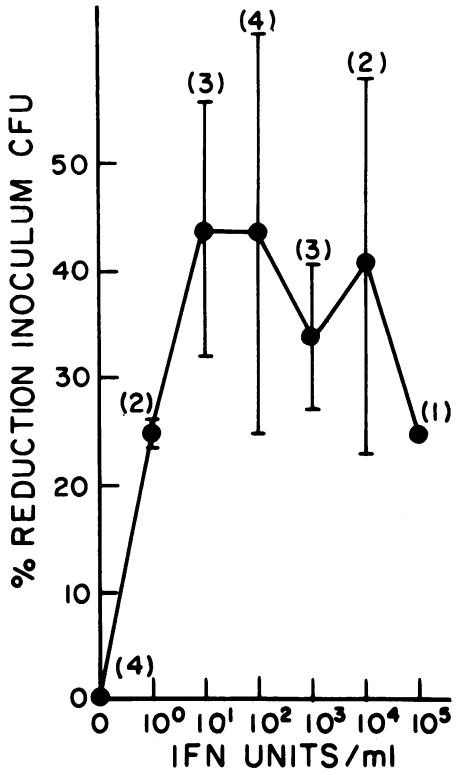

FIG. 1. Effect of IFN on killing of *C. albicans* by macrophages. The mean percent reduction of inoculum CFU ± standard deviation in separate experiments (number of experiments is given in parentheses) by macrophages treated overnight with increasing doses of IFN is shown.

RESULTS

Activation of macrophages for fungicidal activity with IFN. When resident peritoneal macrophages were treated overnight with increasing doses of recombinant gamma-IFN it was found in three experiments that 10 U/ml was sufficient to induce significant ($P < 0.01$) killing (43 ± 10%) of phagocytizable *C. albicans* (Fig. 1). An increase in the IFN concentration 10- to 1,000-fold failed to increase the mean percentage of killing (Fig. 1). In concurrent experiments, it was found that a greater IFN concentration (100 to 10,000 U/ml) was necessary to activate macrophages for significant ($P < 0.01$) killing of *B. dermatitidis* (37 ± 4%), which were nonphagocytizable 8- to 12-μm-diameter cells (usually two or more cells per U) (Fig. 2). Treatment of macrophages with LPS (0.001 to 10 μg/ml) did not have this effect (data not shown). Microscopic examination of cocultures showed that after 4 h of incubation macrophages completely surrounded, and formed distinct clumps around, units of *B. dermatitidis*. These results indicate that the concentration of IFN required to activate resident peritoneal macrophages for significant killing of nonphagocytizable *B. dermatitiditis* is at least 100-fold greater than that required for killing of phagocytizable *C. albicans*.

Effect of antibody to IFN on macrophage activation. When IFN plus antibody to murine IFN was used to treat macrophages, the ability of IFN to activate macrophages for candidacidal activity was neutralized, e.g., 41 versus 10%

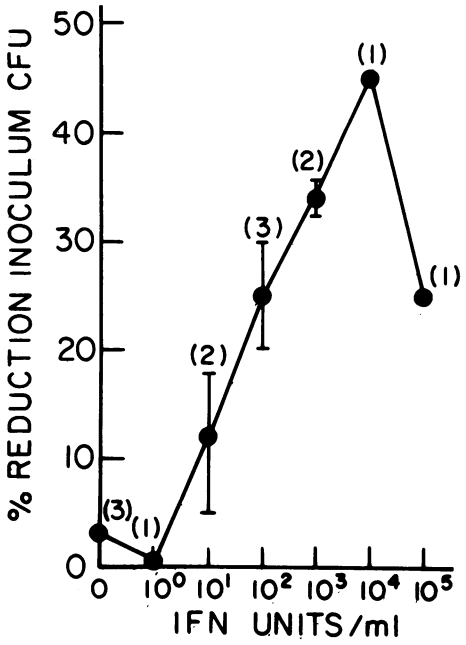

FIG. 2. Effect of IFN on killing of *B. dermatitidis* by macrophages. The mean percent reduction of inoculum CFU ± standard deviation in separate experiments (number of experiments is given in parentheses) by macrophages treated overnight with increasing doses of IFN is shown.

killing (Table 1). Antibody alone had no effect on macrophages (49) (Table 1). Anti-IFN antibody had a similar effect in neutralizing the activity of IFN (10,000 U/ml) for activating treated macrophages to kill *B. dermatitidis* (38 versus 4%; data not shown). These results indicate that IFN was the major factor responsible for inducing fungicidal activity in treated macrophages.

Effect of supernatants on macrophage candidacidal activity. When macrophages were treated with supernatants from ConA-stimulated spleen cells they acquired significant ($P < 0.01$) candidacidal activity (41 versus 0%); however, the ConA (5 μg/ml) control had similar effects. To separate the effect of ConA from the effects of putative lymphokines in supernatants, ConA activity was inhibited with aMM. It is shown in Table 2 that the macrophage-activating effect of ConA was completely inhibitable with 50 mM aMM, e.g., 51 versus 0% killing. On the other hand, aMM did not abrogate the ability of ConA supernatants to induce significant ($P < 0.001$) candidacidal activity (32%) in macrophages (Table 2). These results demonstrate that ConA supernatants contain factor(s) capable of activating macrophages, in addition to ConA.

In another experiment, when macrophages were treated with 0.1 ml of ConA supernatant, 0.01 ml of aMM (0.5 M) and 0.05 ml of antibody to IFN, candidacidal activity was reduced from 38% to a nonsignificant level (17%; $P > 0.05$; data not shown). Collectively, these results indicate that supernatants from ConA-stimulated spleen cell cultures contain a macrophage-activating factor(s) which could be largely neutralized by antibody to murine gamma-IFN.

ConA activation of monolayers for candidacidal activity. When monolayers were incubated overnight with increasing doses of ConA, 1 μg/ml induced significant ($P < 0.01$) killing (28%), and this increased to 56% when the concentration was increased to 10 μg/ml (Fig. 3). Higher concentrations of ConA (50 and 100 μg/ml) failed to produce this effect (Fig. 3). When monolayers were treated with ConA and then ConA was removed, their candidacidal activity was not inhibited if aMM was added at the time of challenge with *C. albicans* (Table 3). For example, ConA-activated monolayers killed 44% of the *C. albicans* inoculum in the presence of 100 mM aMM (Table 3). In contrast to activation by overnight treatment, monolayers could not be activated to kill *C. albicans* by adding ConA at the time of challenge (data not shown), nor did the monolayers kill *C. albicans* which had been incubated with ConA (Table 3). These results indicate that monolayer candidacidal activity is not a lectin-dependent type of cytotoxicity, but rather an activated macrophage type of killing.

Mechanism of ConA activation of macrophages in monolayers. Two possible mechanisms of ConA activation of macrophages in monolayers were investigated: (i) direct action of ConA by binding to macrophages (16, 51) or (ii) ConA stimulation of lymphocytes present in monolayers (10% of total cells) resulting in the production of lymphokines. When monolayers were treated with anti-T-cell serum plus complement and then incubated overnight with ConA, they exhibited no candidacidal activity, whereas if MEM was substituted for anti-T-cell serum or complement or both and incubated with ConA, inoculum CFUs were reduced by 33%. On the other hand, the addition of as few as 25×10^3

TABLE 1. Effect of treatment with anti-IFN antibody on macrophage activation

C. albicans +	CFU per well[a]	Reduction (%) in inoculum CFU	*P*
(Inoculum, 0 h)	833 ± 67		
CTCM (2 h)	1,111 ± 23	<0	NS[b]
Mϕ[c] + CTCM	1,130 ± 137	<0	NS
Mϕ + IFN[d]	493 ± 120	41	<0.01
Mϕ + IFN + Ab[e]	753 ± 49	10	NS
Mϕ + Ab	1,192 ± 112	<0	NS

[a] Mean CFU ± standard deviation of quadruplicate cultures.
[b] NS, Not significant ($P > 0.05$); Student's *t* test.
[c] Resident peritoneal macrophages (Mϕ) were incubated overnight with the indicated materials, culture fluids were removed, and monolayers were challenged for 2 h with *C. albicans*.
[d] Gamma-IFN, 1,500 U/ml of CTCM.
[e] Antibody to IFN gamma.

TABLE 2. Effect of ConA and ConA supernatants on macrophage candidacidal activity

Treatment of Mϕ[a]	CFU per well[b]	Reduction (%) in inoculum CFU[c]	*P*
CTCM	1,030 ± 96	<0	
ConA (5 μg/ml of CTCM)	423 ± 140	51	<0.01
ConA (5 μg/ml of CTCM) + aMM (50 mM)	1,100 ± 149	<0	NS[d]
ConA supernatant[e]	590 ± 26	32	<0.001
ConA supernatant + aMM (50 mM)	535 ± 79	38	<0.001

[a] Resident peritoneal macrophages (Mϕ) were incubated overnight with the indicated materials, culture fluids were removed, and monolayers were challenged for 2 h with *C. albicans*.
[b] Mean CFU ± standard deviation of quadruplicate cultures after 2 h.
[c] Inoculum CFU, 863 ± 65 CFU per well at time zero.
[d] NS, Not significant ($P > 0.05$); Student's *t* test.
[e] Supernatant from spleen cell cultures stimulated with ConA (5 μg/ml of CTCM) for 1 day.

lymph node cells per monolayer, followed by incubation overnight with ConA (5 μg/ml), resulted in enhanced monolayer candidacidal activity, e.g., up to 80% (data not shown). These data do not support the direct activation of macrophages for candidacidal activity by ConA, but rather activation by products (lymphokines) from ConA-stimulated lymphocytes.

Effect of supernatants on macrophage killing of *B. dermatitidis*. Supernatants from spleen cells cultured with or without ConA (5 μg/ml) were tested for their ability to activate resident peritoneal macrophages for fungicidal activity against *B. dermatitidis*. Supernatants from cultures stimulated with ConA for 1 day activated macrophages for significant ($P < 0.01$) killing (30 ± 4%) (Fig. 4). Supernatants from cultures stimulated for 2 or 3 days with ConA were less effective (Fig. 4). This effect was not seen when macrophages were treated with CTCM, supernatants from nonstimulated spleen cell cultures, ConA (5 μg/ml), or LPS (0.001 to 10 μg/ml) (data not shown).

When macrophages were treated with ConA supernatants plus anti-IFN antibody, the activation of macrophages for the ability to kill *B. dermatitidis* was reduced from 32 to 12% (Table 4). These findings demonstrate that the activating factor in ConA supernatants was inhibitable with anti-IFN antibody and indicate that the principal activating factor is IFN.

TABLE 3. Effect of ConA on candidacidal activity of macrophages

Treatment of Mϕ[a]	Challenge[b]	CFU per well[c]	Reduction (%) in inoculum CFU	*P*
CTCM	*C. albicans* + aMM	1,250 ± 130	<0	NS[d]
ConA	*C. albicans* + aMM	486 ± 90	44	<0.001
CTCM	*C. albicans* treated with ConA	1,567 ± 180	<0	NS
ConA	*C. albicans* treated with ConA	730 ± 80	25	<0.01

[a] Resident peritoneal macrophages (Mϕ) were incubated overnight with the indicated materials, culture fluids were removed, and monolayers were challenged for 2 h with *C. albicans*. ConA, 5 μg/ml.
[b] *C. albicans* inoculum was 863 ± 65 CFU/ml at time zero (100 mM aMM). *C. albicans* treated with ConA (*C. albicans* was incubated with 5 μg of ConA per ml and then washed) was 980 ± 60 CFU/ml at time zero.
[c] Mean CFU ± standard deviation of quadruplicate cultures at 2 h.
[d] NS, Not significant ($P > 0.05$; Student's *t* test).

DISCUSSION

We report here that IFN induces fungicidal activity in resident peritoneal macrophages against *C. albicans* and *B. dermatitidis*. These new findings suggest a role for IFN in cellular resistance to these pathogens. This concept is supported by the findings of Wu-Hsieh et al. (55), which showed that gamma-IFN-like factor(s) in ConA-stimulated T-cell

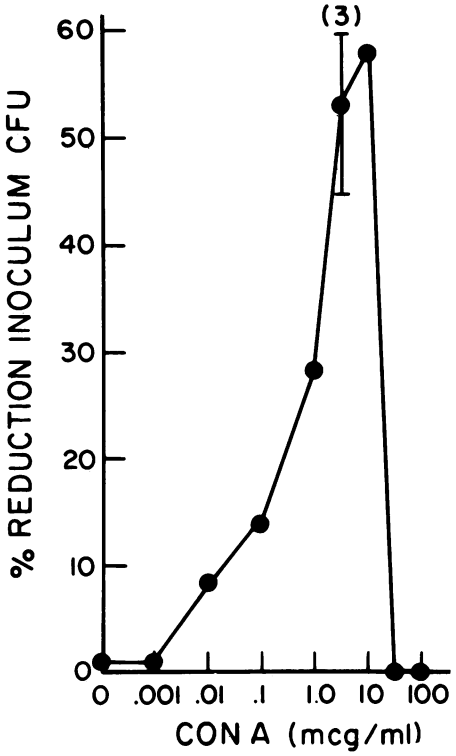

FIG. 3. Effect of ConA treatment of macrophage monolayers on killing of *C. albicans*. The mean percent reduction of inoculum CFU ± standard deviation from three experiments, or a single experiment, by monolayers treated overnight with increasing concentrations of ConA is shown. mcg/ml, Micrograms per milliliter.

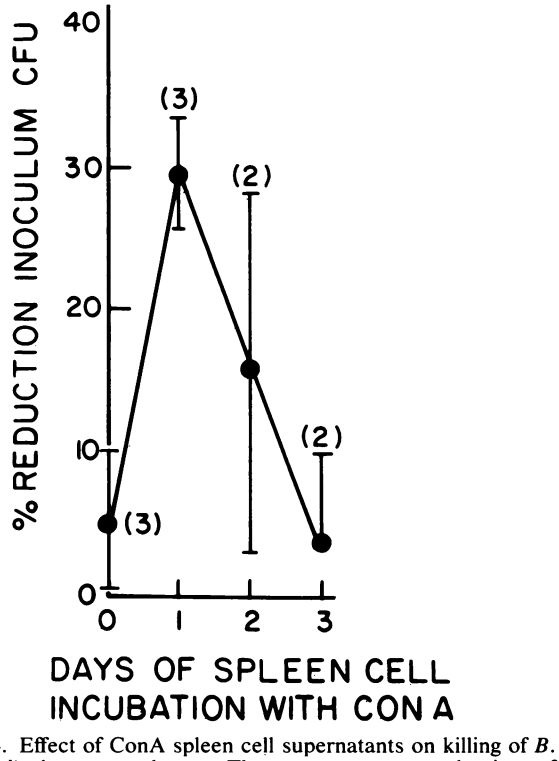

FIG. 4. Effect of ConA spleen cell supernatants on killing of *B. dermatitidis* by macrophages. The mean percent reduction of inoculum CFU ± standard deviation in experiments (number of experiments is given in parentheses) by macrophages treated overnight with supernatants from spleen cell cultures stimulated with ConA for 1, 2, or 3 days is shown.

TABLE 4. Effect of anti-IFN antibody on ConA supernatant activation of macrophages

B. dermatitidis +	CFU per well[a]	Reduction (%) inoculum CFU	*P*
(Inoculum 0 h)	305 ± 47		
CTCM (4 h)	405 ± 64	<0	NS[b]
Mϕ[c] + CTCM	323 ± 35	<0	NS
Mϕ + ConA supernatant[d]	207 ± 62	32	0.05
Mϕ + ConA supernatant + anti-IFN[e]	270 ± 29	12	NS
Mϕ + ConA (5 μg/ml)	313 ± 35	<0	NS

[a] Mean CFU ± standard deviation of quadruplicate cultures.
[b] NS, Not significant ($P > 0.05$); Student's *t* test.
[c] Resident peritoneal macrophages were incubated overnight with the indicated material, culture fluids were removed, and monolayers were challenged for 4 h with *B. dermatitidis*.
[d] Supernatant from spleen cells stimulated for 1 day with ConA (5 μg/ml).
[e] Antibody against IFN, 0.05 ml per well.

hybridoma supernatants enabled resident peritoneal macrophages to restrict intracellular replication of *H. capsulatum* in vitro. Furthermore, activation of resident peritoneal macrophages for killing of *Coccidioides immitis* by lymphokines in supernatants from antigen-stimulated spleen cells from immunized mice has been reported (3); however, the presence of IFN in active supernatants was not assessed.

An unexpected finding in this study was the different concentrations of IFN required to activate macrophages for killing phagocytizable *C. albicans* (10 U/ml) as opposed to nonphagocytizable *B. dermatitidis* (100 to 10,000 U/ml). The dose of IFN required to activate resident peritoneal macrophages for candidacidal activity was in the same range (1 to 10 U/ml) as that required for induction of nonphagocytic tumoricidal activity in elicited murine peritoneal macrophages (39, 49) or human peripheral blood monocytes (26, 27). On the other hand, the dose of IFN necessary to activate human monocyte-derived macrophages (100 to 300 U/ml for 3 days) to kill intracellular *Toxoplasma gondii* (36, 37) was comparable to that (100 to 1,000 U/ml for 1 day) required to activate resident peritoneal macrophages to kill *B. dermatitidis*. The relationship between the concentration of IFN and the induction of different microbicidal and tumoricidal mechanisms is not clear at this time.

LPS (0.001 to 10 μg/ml) failed to induce a significant ability to kill *C. albicans* or *B. dermatitidis* in resident peritoneal macrophages. This ruled out the possibility that the activity measured in IFN preparations was due to contamination with LPS. The inability of LPS to activate macrophages in vitro for fungicidal activity is in sharp contrast to the capacity of LPS to activate murine peritoneal macrophages (15, 50) or human monocytes (11) for the killing of tumor cell targets. Recently it has been shown that macrophages in different stages of activation may be both tumoricidal and microbicidal, only tumoricidal, or only microbicidal (12, 54). Furthermore, Sverdersky et al. (49) have shown that anti-IFN antibody did not affect the activation of macrophages by LPS for tumoricidal activity.

We show here that the macrophage-activating ability of supernatants from ConA-stimulated spleen cell cultures could be neutralized with anti-gamma-IFN antibody. Similarly, IFN has been identified as the active factor in supernatants of other systems for activating macrophages, e.g., for cytotoxicity (26, 30, 49) and microbicidal functions (36, 53). Our findings extend the range of microbicidal activity of IFN-activated macrophages to *C. albicans* and *B. dermatitidis*. The role of naturally produced IFN in host defenses against these fungi merits further investigation. Recombinant DNA-produced IFN may have a therapeutic role in fungal diseases.

Treatment of monolayers with ConA resulted in significant candidacidal activity. This was shown to be associated with the lymphocytes present in the monolayers and not by the direct action of ConA on macrophages. Although activation of macrophages in vitro by ConA for cytotoxicity has been reported previously (51, 52), the radical differences in these cidal systems may account for the discordance.

In contrast to candidacidal activity of ConA-treated monolayers, *B. dermatitidis* was not killed by these cells. A reasonable explanation for this apparent discrepancy is based on our finding that 10 to 100 times more IFN was required to activate macrophages to kill *B. dermatitidis*, and this IFN level was not attained in ConA-treated monolayer cultures.

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