Candidacidal Activity of Mouse Macrophages In Vitro

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Mouse peritoneal macrophages were infected in vitro with *Candida albicans*, and the phagocytic and candidacidal activities were estimated by microscopic examination of Giemsa-stained cells. Activated macrophages obtained from either BCG-vaccinated animals or by in vitro exposure of normal macrophages to phytohemagglutinin-induced lymphokines exhibited higher phagocytic and candidacidal activities than did normal macrophages. However, activated macrophages obtained by in vitro exposure of macrophages to candida-induced lymphokines exhibited the highest phagocytic and candidacidal activities. The incorporation of immune mouse serum into the culture medium also enhanced the phagocytic and candidacidal activities of the normal macrophages but failed to improve the function of the activated macrophages. These results suggest that both activated macrophages and antibodies may be required for controlling candida infections in mice.

The mechanisms of immunity in candidiasis are not clearly understood; extensive data are available from both clinical and experimental studies, but the results are contradictory. The clinical studies have clearly established the importance of a cell-mediated immune response in chronic mucocutaneous candidiasis, a unique form of the disease often associated with a T-cell defect (3, 11). However, experimental studies have presented conflicting reports regarding the role of the cell-mediated immune response (20, 21; D. K. Giger and J. E. Domer, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, F42, p. 125). It has been shown that passive transfer of immune serum protects animals against challenge with candida (15, 17), and evidence is available that polymorphonuclear neutrophils possess anticandidal activity (12). However, the information regarding the function of macrophages is not clear. Macrophages play an essential role as effector cells in both humoral and cell-mediated immunity (13), and several studies have documented the importance of mononuclear phagocytes in the immune response to obligate intracellular organisms (8, 25). It has been shown that mice infected with *Candida albicans* exhibit enhanced reticuloendothelial system phagocytosis as measured by the clearance of colloidal carbon (1). Others have shown that normal macrophages have the potential for the phagocytosis of *C. albicans*, but this may not be sufficient to control the infection (16). It has also been shown that rabbit alveolar macrophages can effectively inhibit the growth of *C. albicans* in vitro (18). The purpose of this study was to examine the interaction of *C. albicans* with normal and activated mouse macrophages and to

determine the influence of antibody on their function.

MATERIALS AND METHODS

Animals. Swiss albino mice of either sex weighing 20 to 25 g were obtained from the Experimental Animal Facility, All India Institute of Medical Sciences, New Delhi.

Microorganisms. A strain of *C. albicans* obtained from the departmental stock collection was passaged regularly on Sabouraud dextrose agar. The yeast cells were grown on Sabouraud dextrose agar for 24 to 48 h at 37°C, harvested, and washed with sterile saline. The cells were harvested in Hanks balanced salt solution instead of saline when used for in vitro infection.

The Danish strain of BCG (no. 1331) was supplied as a lyophilized preparation (BCG Vaccine Laboratory, Guindy, Madras) and suspended in sterile saline before use.

A strain of *Listeria monocytogenes* obtained from the departmental stock collection was passaged regularly on brain heart infusion broth used as a test organism for the assessment of bactericidal potential of macrophages in vitro.

Preparation of the macrophage monolayer. The peritoneal cells were obtained from mice after the intraperitoneal injection of 5 ml of Hanks balanced salt solution (Bios Laboratory, Bombay) containing 10 U of preservative-free heparin (Bios Laboratory) per ml. The peritoneal washing was withdrawn through the abdominal wall with a 20-gauge needle and centrifuged at 300 × g for 10 min. The peritoneal cells were adjusted to 10⁶ cells per ml in tissue culture medium (TC 199) with Earle salt (Bios Laboratory) supplemented with 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid) buffer (Centron Research Laboratory, Bombay)-10% fetal calf serum (Difco Laboratories, Detroit, Mich.)-100 U of penicillin per ml-100 µg of streptomycin per ml. The cell suspension (1 ml) was placed in a Leighton tube with

a cover slip and incubated for 24 h at 37°C. After 24 h, the nonadherent cells were removed by washing, and fresh medium was added.

Preparation of the macrophage monolayer from BCG-vaccinated animals. Peritoneal exudate cells were harvested on day 22 from the mice vaccinated intravenously with BCG (2×10^7 colony-forming units) on days 0, 7, and 21 and cultured in the same manner as described above.

Preparation of the macrophage monolayer from immunized animals. Mice were immunized intravenously with 3×10^3 *Candida* cells on days 0 and 15, and the peritoneal exudate cells were harvested after 10 days and cultured in the same manner as described above. The macrophages from the immunized animals were considered to be immune macrophages and the lymphocytes were considered to be immune lymphocytes.

Preparation of splenic lymphocyte culture supernatants. The spleens were removed from the normal animals, minced, and pressed through 80-mesh stainless-steel sieves. The cell suspension was washed twice with TC 199 and finally suspended to a concentration of 2×10^6 cells per ml in TC 199 supplemented with 25 mM HEPES buffer-10% fetal calf serum-300 μ g of glutamine per ml-100 U of penicillin per ml-100 μ g of streptomycin per ml. The cells were incubated with 20 μ g of phytohemagglutinin (PHA; Difco Laboratories) per ml for 48 h at 37°C, and the culture supernatant was obtained by centrifugation at $200 \times g$ for 10 min. This culture supernatant was referred to as lymphokines. The concentration of PHA was determined by a preliminary dose-response assay. Spleen cells were also incubated without PHA in the same manner to obtain the control culture supernatant.

The candida-induced lymphokines were prepared in the same manner by incubating the immune lymphocytes with 50 μ g of the candida antigen dermatophytin O (Hollister-Stier Laboratories, Spokane, Wash.) per ml for 48 h at 37°C. Control supernatants were prepared after the incubation of lymphocytes without the candida antigen.

Activation of macrophages by splenic lymphocyte culture supernatants. Macrophage monolayers were incubated with 1 ml of PHA-induced lymphokines for 48 h at 37°C and considered to be PHA-activated macrophages. The control macrophage monolayers were incubated with control lymphocyte culture supernatants in the same manner.

The candida-activated macrophages were prepared by incubating the immune macrophages with candida-induced lymphokines, and the macrophage monolayers were incubated with control culture supernatants for the control macrophages.

Listericidal assay. For assessing the state of activation achieved by these procedures, a listericidal assay was used (4). The inocula of *L. monocytogenes* were prepared from a 16-h culture and washed three times with phosphate-buffered saline. The organisms were suspended in TC 199 containing 10% fetal calf serum without antibiotics to achieve a multiplicity of infection of approximately five organisms to one macrophage when 1 ml was added to the macrophage monolayer. The phagocytosis was allowed to proceed for 90 min at 37°C, and then monolayers were washed

with phosphate-buffered saline to remove extracellular bacteria and further incubated with fresh media. The cover slips were removed at 6 and 24 h after phagocytosis, rinsed with phosphate-buffered saline, fixed in 2% glutaraldehyde, stained in Jenner-Giemsa stain, and observed under a microscope.

Production of antisera. Anticandidal antibody was obtained from mice immunized with two intraperitoneal injections, followed by two intravenous injections, on days 0, 7, 14, and 21 with 0.5 ml of formalinized candida cells. Thus, each animal received a total of 2 ml of suspension containing 4×10^7 cells. One week after the last injection, the animals were bled by puncturing the retroorbital plexus, and sera were collected and stored at -20°C until use.

The antibody titer of the pooled serum was determined by an agglutination test (24) to be 1:512.

Effects of immune serum. Normal mouse serum or immune mouse serum (IMS) was incorporated into TC 199 containing 10% fetal calf serum, and *C. albicans* was suspended in this medium before infection of the monolayer. After phagocytosis, the monolayers were further incubated with this medium.

Infection of the macrophage monolayer. The culture medium was removed by aspiration, and the monolayer was infected with approximately 10^6 cells of *C. albicans* in 1 ml of TC 199 containing 10% fetal calf serum. The phagocytosis was allowed to proceed for 30 min at 37°C, and then the monolayer was washed with TC 199 to remove extracellular yeast cells and further incubated with fresh medium. The cover slips were removed after 4 h of infection, rinsed with phosphate-buffered saline, fixed with 2% glutaraldehyde, stained with Jenner-Giemsa stain, and observed under a microscope.

Estimation of phagocytic and candidacidal activities. The stained cells were examined microscopically to determine the percentage of macrophages showing phagocytosis and the number of intracellular candida with and without germ tubes. A total of 200 stained cells were counted in each sample.

Candida that were incapable of producing germ tubes at 4 h postinfection were considered to be non-viable. That the lack of germ tube formation was indeed an index of candidacidal activity was confirmed by the candidacidal assay (12).

Candidacidal assay. After 4 h of infection, the cover slips were removed, the macrophages were lysed with 2.5% sodium deoxycholate, and the viability of candida was tested by the methylene blue dye exclusion technique.

Statistical analysis. The chi-square test was applied to analyze the data on the relative frequency of the intracellular yeast cells producing germ tubes and those without germ tubes in the test and control groups.

RESULTS

The fate of *C. albicans* in normal mouse peritoneal macrophages was studied with different ratios of candida to macrophages. Based on these preliminary experiments, a candida/macrophage ratio of 1:1 was found suitable for subsequent experiments. The period of observation

was restricted to 4 h as with a higher proportion of candida and at a longer period of incubation, there was too much germ tube formation, making it difficult to record the microscopic observations.

Listericidal assay. Direct microscopic observation by the method of Cole (4) was applied to assess the nonspecific antimicrobial activity of these three different types of activated macrophages, i.e., BCG-, PHA-, and candida-activated, after infection with *L. monocytogenes* at a 5:1 ratio of listeria to macrophages. All three types of activated macrophages inhibited the growth of intracellular listeria, whereas normal macrophages supported the growth of intracellular bacteria and were ultimately destroyed by the bacterial overgrowth within 24 h postinfection.

Effect of BCG activation of macrophages on phagocytic and candidacidal activities. The BCG-activated macrophage monolayer was prepared from the peritoneal exudate cells of the mice after repeated BCG vaccinations. When these activated macrophages were infected with *C. albicans*, 54 to 58% of the macrophages ingested yeast cells, in comparison with 45 to 48% of normal macrophages. The intracellular yeast cells producing germ tubes amounted to 33 to 43% in activated macrophages, in comparison with 53 to 58% in normal macrophages. The difference in the germ-producing cells between the two groups was statistically significant (Table 1).

Effect of PHA-induced lymphokine activation on phagocytic and candidacidal activities. A spleen cell culture supernatant or control preparation was applied to a normal macrophage monolayer for 48 h at 37°C, and the activated and control macrophages were obtained. When they were infected with *C. albi-*

cans, 43 to 48% of activated macrophages phagocytized candida, in comparison with 40 to 46% of control macrophages. Germ tube formation was seen in 32 to 38% of the intracellular yeast cells in activated macrophages, as compared with 55 to 56% in control macrophages (Table 2).

Effect of BCG activation and PHA activation on candidacidal activity. The candidacidal assay was performed to determine the live and dead intracellular yeast cells by methylene blue dye exclusion at 4 h postinfection. It was shown that about 40 to 45% of the candida cells were found viable in both BCG- and PHA-activated macrophages, in comparison with 56 to 62% in control macrophages (Table 3). Thus, the number of germ tube-producing cells corresponded to the number of viable cells.

Effect of candida-activated macrophages on phagocytic and candidacidal activities. Immune macrophages and immune lymphocytes were obtained from mice immunized with *C. albicans*. The immune macrophages incubated with candida induced lymphokines to obtain the candida-activated macrophages. When both activated and control macrophages were infected with *C. albicans*, 78 to 87% of the former and 46 to 55% of the latter ingested yeast cells. Furthermore, 20 to 30% of the ingested yeast cells produced germ tubes in activated macrophages, in comparison with 60 to 61% in control macrophages (Table 4).

Effect of antibody on macrophage function. When 2% normal mouse serum or 2% IMS was incorporated into the medium and the monolayer was infected with candida, it was found that a larger number of macrophages ingested yeast cells and a smaller number of ingested yeast cells produced germ tubes in the presence of IMS (Table 5). It was also observed

TABLE 1. Fate of *C. albicans* in BCG-activated mouse macrophages in vitro

Expt	Macro- phage ^a	Time in- terval (h)	Percentage of macrophages showing phagocytosis	No. of candida per 100 macrophages showing phagocytosis			
				Total	With germ tubes ^b	Without germ tubes	Statistical analy- sis (<i>P</i> value)
1.	A	4	54	150	50 (33)	100	<0.001
	C		45	130	75 (58)	55	
2.	A	4	58	210	84 (40)	126	<0.001
	C		48	190	108 (57)	92	
3.	A	4	56	180	76 (42)	104	<0.01
	C		45	160	90 (56)	70	
4.	A	4	56	200	70 (39)	130	<0.005
	C		46	190	100 (53)	90	

^a A, Activated macrophage; C, normal macrophage.

^b Values in parentheses indicate percentages.

TABLE 2. Fate of *C. albicans* in PHA-activated mouse macrophages in vitro

Expt	Macro-phage ^a	Time interval (h)	Percentage of macrophages showing phagocytosis	No. of candida per 100 macrophages showing phagocytosis			
				Total	With germ tubes ^b	Without germ tubes	Statistical analysis (P value)
1.	A	4	50	190	76 (40)	114	<0.001
	C		40	220	136 (62)	84	
2.	A	4	49	170	65 (38)	105	<0.005
	C		45	160	96 (60)	64	
3.	A	4	48	175	65 (38)	110	<0.001
	C		40	180	104 (58)	76	
4.	A	4	53	150	65 (42)	85	<0.05
	C		46	166	80 (55)	76	

^a See Table 1, footnote a.^b See Table 1, footnote b.TABLE 3. Candidacidal assay with normal and non-specifically activated mouse peritoneal macrophages in vitro^a

Expt	Percentage of live cells ^b in:			
	Normal macrophages	BCG-activated macrophages	Control macrophages	PHA-activated macrophages
1	60	40	58	40
2	58	45	56	46
3	56	42	58	42
4	62	43	57	44
Mean percentage $\pm$ SD ^c	59.0 $\pm$ 2.58	42.5 $\pm$ 2.08	57.25 $\pm$ 0.96	43.0 $\pm$ 2.58

^a The P value for normal and BCG-activated macrophages and for control and PHA-activated macrophages was <0.001.^b Live cells were determined by the methylene blue dye exclusion technique at 4 h postinfection.^c SD, Standard deviation.

that preincubation of the macrophage monolayer with 2% IMS before infection with *C. albicans* did not enhance the macrophage function. A similar observation was made when activated, instead of normal, macrophages were used and when 2% IMS was incorporated into the medium.

DISCUSSION

The observations presented here suggest that both cellular and humoral responses play a role in facilitating the intracellular killing of *C. albicans* by mouse macrophages.

It is well established that in infectious diseases, cell-mediated immune response operates mostly through macrophages. The activation of macrophages, which is accompanied by an enhanced listericidal activity, is an inducible phenomenon mediated by lymphokines (5, 22). The

TABLE 4. Fate of *C. albicans* in candida-activated mouse macrophages in vitro^a

Expt	Source of lymphocytes	Source of macrophages	Percentage of macrophages showing phagocytosis	No. of candida per 100 macrophages showing phagocytosis		
				Total	With germ tubes ^b	Without germ tubes
1. A	Normal	Normal	55	180	110 (61)	70
	B	Immune	87	225	45 (20)	180
	C	Normal	56	180	102 (56)	78
	D	Immune	60	220	90 (41)	130
2. A	Normal	Normal	46	177	107 (60)	70
	B	Immune	78	225	70 (30)	155
	C	Normal	50	190	114 (60)	76
	D	Immune	55	225	100 (44)	125
3. A	Normal	Normal	48	190	114 (60)	76
	B	Immune	82	262	55 (21)	207
	C	Normal	55	160	100 (62)	60
	D	Immune	60	180	82 (45)	98

^a Time interval, 4 h. Statistical analysis: A vs B = P < 0.001; C vs D = P < 0.05; B vs D = P < 0.005; A vs C = P > 0.05; A vs D = P < 0.001.^b See Table 1, footnote b.

lymphokines are liberated either upon the exposure of sensitized lymphocytes to the antigen (14, 23) or upon the exposure of normal lymphocytes to phytomitogens (7). BCG has also been extensively used for the activation of macrophages (2, 19).

We investigated the interaction of activated macrophages with *C. albicans* under different conditions, including in vitro lymphokine activation by PHA, as well as by the candida antigen, and in vivo BCG activation. These activated macrophages showed increased listericidal activity and handled *C. albicans* more efficiently.

Both the phagocytic and candidacidal activities were enhanced in the non-specifically acti-

TABLE 5. Effect of IMS on the fate of *C. albicans* in normal mouse macrophages in vitro

Expt	Treatment ^a	Time interval (h)	Percentage of macrophages showing phagocytosis	No. of candida per 100 macrophages showing phagocytosis			
				Total	With germ tubes ^b	Without germ tubes	Statistical analysis (P value)
1.	C	4	40	170	100 (58)	70	<0.001
	T		54	170	63 (37)	107	
2.	C	4	45	178	108 (56)	70	<0.001
	T		57	180	72 (39)	118	
3.	C	4	48	180	105 (54)	75	<0.001
	T		55	180	72 (40)	108	
4.	C	4	43	170	95 (56)	75	<0.001
	T		57	188	64 (35)	124	
5.	C	4	46	170	95 (56)	75	<0.001
	T		60	190	66 (35)	124	

^a C, Normal macrophages infected with *C. albicans* suspended in 2% normal mouse serum; T, normal macrophages infected with *C. albicans* suspended in 2% IMS.

^b See Table 1, footnote b.

vated macrophages by PHA-induced lymphokines. Similar results were achieved with activated macrophages obtained from BCG-vaccinated mice. However, the highest phagocytic and candidacidal activities were seen in specifically activated macrophages after the exposure of immune macrophages to candida-induced lymphokines. Still, even after this method of activation, about 20 to 30% of the intracellular candida cells produced germ tubes.

This dichotomy in the function of non-specifically and specifically activated macrophages has also been shown in other studies (4, 19). The mechanism of specific activation as different from nonspecific activation is not known. But it could be caused by the release of T-cell receptors either alone or complexed with the antigen. It could also be due to antibodies as spleen cells contributed both T and B lymphocytes. For studying the last possibility, the experiments were planned to incorporate candida antibodies in the culture medium before infection of the monolayer and incubated further with this medium. Normal mouse serum was incorporated into the medium as a control. It has been shown that the growth of candida was substantially modified in the normal macrophages after the interaction of antibody with candida; only 35 to 40% of the intracellular yeast cells were capable of producing germ tubes, in comparison with 54 to 58% in the controls. But the number of germ tube-producing cells was not reduced in the activated macrophages by the interaction of candida with antibody. The antibody was opsonic, and not cytophilic, in nature, as indicated by the fact that the preincubation of the normal mac-

rophages with IMS did not enhance the function of the macrophages. Attempts were also made to preincubate *C. albicans* with IMS, but such procedures quickly produced germ tubes.

The mechanisms by which the normal macrophages suppressed the growth of *C. albicans* after the interaction of antibody with candida is not known. But reports are available in which it has been shown that *Toxoplasma gondii* opsonized with immune serum is readily destroyed by normal macrophages, whereas unopsonized toxoplasmas escape the microbicidal activity of macrophages (9, 10). Similar findings have also been reported in rickettsiae (6). Survival of the organisms in the macrophages has been shown to be due to the failure of lysosomes to fuse with phagosome-containing organisms (10). Some recent data suggest that the organism itself is responsible for nonfusion and that humoral antibodies to components of the organism allow the fusion to occur (6, 9, 10). Whether in the candida-macrophage system the antibody operates through a similar mechanism has not been investigated.

The present study has shown the ability of the activated macrophages to suppress the growth of *C. albicans* in the absence of antibody and that the function of normal macrophages is enhanced after candida-antibody interaction.

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