INVOLVEMENT OF HOST MACROPHAGES IN THE IMMUNOADJUVANT ACTIVITY OF AMPHOTERICIN B IN A MOUSE FUNGAL INFECTION MODEL

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We have recently reported the *in vivo* augmentation of resistance to experimental *Candida albicans* injection by amphotericin B in mice and have shown that this event is concurrent with the appearance in the spleen of a highly candidacidal cell population reactive *in vitro* against ^{51}Cr-labeled yeast cells. In the present study we characterize these *in vitro* fungicidal effectors as macrophages and describe the conditions of amphotericin B treatment most suitable for inducing candidacidal activity. We also report that macrophages from intact mice can be activated *in vitro* to become cytotoxic against *Candida*. The possible mechanisms through which the amphotericin B activated macrophages exert their increased anti-*Candida* activity are also investigated.

We have previously shown that amphotericin B (AmB) can, under selected experimental conditions, protect mice against systemic challenge with *Candida albicans* by means of a mechanism largely independent of its chemotherapeutic antifungal activity[1]. Because of the well known "adjuvant-like" properties of AmB[2,3], we started to examine its effects on isolated components of the host defense system, particularly those with candidacidal activity[4,5]. In the present paper, we report on the activation of splenic macrophages by *in vivo* or *in vitro* exposure to AmB, and define the experimental conditions suitable for achieving optimal anti-*Candida* immunoadjuvant effects. Studies are also described aimed at clarifying the cellular mechanisms through which AmB confers strong anti-*Candida* activity on macrophages. This effect apparently involves a more rapid ingestion of the yeast combined with greater phagocytic ability of individual macrophages; on incubation of these effectors with live *Candida*, marked inhibition of hyphae formation is observed.

Materials and Methods

Mice

Hybrid (BALB/c Cr×DBA/2) F1 (CD2F1: H-2^d/H-2^d) mice were obtained from Charles River Breeding Laboratories, Calco, Milan, Italy.

Chemicals

AmB (Fungizone) kindly supplied by E. R. Squibb & Sons, Inc., Princeton, N. J., was provided in vials containing 50 mg of AmB and 41 mg of sodium deoxycholate with 25.2 mg of sodium phosphate as a buffer. The drug was dissolved in sterile, non-pyrogenic 5% glucose in H_2O and injected into the mice intraperitoneally (ip) in a volume of 0.1 ml/10 g of body weight. Under these conditions a solution of 1 mg of AmB per ml gave the routinely used dose of 10 mg of AmB per kg.

C. albicans

The *C. albicans* strain (laboratory identification name, CA-6) used throughout this study was isolated from a clinical specimen and identified by MARCONI *et al.*[6] according to the taxonomic criteria of VAN UDEN and BUCKELY[7]. The yeast was grown at 28°C under slight agitation in low-glucose Winge medium composed of glucose 0.2% (w/v) and yeast extract (BBL, Microbiology Systems, Cockeysville, Md.) 0.3% (w/v) until a stationary phase of growth was reached (about 24 hours). Under these conditions, the culture gave a yield of approximately 2.8×10^8 cells per ml, and the organism grew as an essentially pure yeast-phase population. After the 24-hour culture, cells were harvested by low-speed centrifugation ($1,000 \times g$), washed twice in saline, and diluted to the desired density. In the *in vivo* studies, *C. albicans* was injected intravenously *via* the tail vein in a volume of 0.5 ml per mouse.

Cell Fractionation Procedures

(i) Plastic Adherence: Forty million effector cells suspended in a volume of 10 ml of complete RPMI 1640 were incubated for 3 hours at 37°C in a 5% CO_2 atmosphere in 93-mm petri dishes (Nunc, Roskilde, Denmark). At the end of the incubation, the dishes were extensively washed with RPMI 1640 medium to remove the nonadherent cells. The adherent cells were recovered by scraping with a rubber policeman, washed, and resuspended (viability, 80 to 90%) in complete RPMI 1640.

(ii) Nylon Column: Effector cells were passed over a nylon fiber column as previously described[8]. Briefly, the sterile nylon columns were rinsed with 20 ml of RPMI supplemented with 5% fetal calf serum. The columns were drained of excess medium and then replaced in sterile syringe covers and put in a CO_2 incubator at 37°C at least 1 hour before loading of cells. Then 10^8 cells in a volume of 2 ml were added to the column and washed into the nylon wool with 0.5 to 1 ml of warm (37°C) medium. The columns were replaced in the sterile syringe covers and left for 45 minutes at 37°C. The columns were then washed slowly with warm (37°C) medium, the first 25 ml of effluent was collected in 50-ml conical tubes, and the cells were pelletted at $290 \times g$ for 10 minutes at 4°C. Cell recovery was about 30%.

(iii) Carbonyl-iron Powder and Magnet: Removal of phagocytic cells from the effector cell population was performed as previously described[9]. Briefly, 25 ml of spleen cell suspension (10^7 cells per ml) was incubated with 25 mg of carbonyl-iron powder (G.A.F. Corp., New York, N.Y.) in a 50-ml conical tube (Falcon Plastics, Oxnard, CA) for 60 minutes at 37°C. To remove the cells that ingested iron particles, the tube was placed on the top of a magnet, and the supernatant was removed. This last step was repeated six to eight times. The cells were then washed and used as effectors in the microcytotoxicity assays.

(iv) Treatment with Anti-theta(Anti-Thy 1.2) Serum Plus Complement: Monoclonal antibodies to Thy 1.2 were purchased from New England Nuclear Corp., Boston, MA (lot LK 114). Thirty million spleen cells were preincubated with anti-Thy 1.2 antiserum diluted 1 : 100 in complete RPMI 1640 medium for 30 minutes at room temperature, washed once in complete RPMI 1640, suspended in a 1 : 4 dilution of low-toxicity-m rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada), and incubated for 45 minutes at 37°C. The surviving cells were then washed twice and counted. In all experiments a complement control was performed in which the first incubation was in medium alone and the second incubation was with complement. Cell recovery was between 50 and 60%.

(v) Treatment with Anti-Asialo GM1 Antiserum Plus Complement: The antiserum, previously shown to react selectively with mouse NK cells[10] was obtained from Wako Chemicals GmbH, Düsseldorf, West Germany. Spleen cells were treated with a 1/200 dilution of antiserum in RPMI 1640 medium for 30 minutes at room temperature, washed twice, suspended in a 1/4 dilution of low-toxicity-m rabbit complement, and incubated for 1 hour at 37°C. The surviving cells were then washed twice, counted, and tested for residual activity in the microcytotoxicity assays.

Preparation of Effector Cells

For the *in vitro* assays, effector cells from 5 to 10 animals were pooled. Spleen cell suspensions were obtained by standard methods.

^{51}Cr-release Assay against *C. albicans*

The candidacidal activity of various effector cell populations was assessed by means of a previously described method[11]. Briefly, 2×10^8 *C. albicans* cells were incubated for 2 hours at 37°C under 5% CO_2 with 300 μCi of Na_2-$^{51}CrO_4$ (Amersham International Ltd., Amersham, England), washed three times in saline, counted, and resuspended in medium to a density of 5×10^5 cells/ml. Under standard labeling conditions, 5×10^4 *C. albicans* cells gave 2,000$\pm$500 cpm. Various numbers of effector cells in 0.1 ml were mixed in U-shaped 96-well microtiter plates with 5×10^4 radiolabeled *C. albicans* cells in 0.1 ml. After 4 hours of incubation at 37°C under 5% CO_2, the plates were centrifuged at $800\times g$ for 10 minutes. The radioactivity in 0.1 ml of the supernatant was measured on a γ-scintillation counter. The base-line ^{51}Cr-release was that of *C. albicans* incubated alone in complete RPMI 1640 medium, and in no case did it exceed 20% of total counts per minute incorporated by target cells.

Experimental results have been expressed as the percent lysis in the experimental group (quadruplicate samples) above the base-line control by the following formula; specific ^{51}Cr-release (%)=cpm (experimental group)$-$cpm (spontaneous release)/(0.5$\times$total cpm)$\times$100, where total cpm is the radioactivity incorporated by 5×10^4 *C. albicans* cells.

Plate Counts

Phagocytic cells (5×10^5 in 0.1 ml per well) were infected with unlabeled *C. albicans* cells (5×10^4 cells in 0.01 ml per well). After 1 or 4 hours of incubation at 37°C under 5% CO_2, the plates were vigorously shaken, and serial dilutions were made in distilled water from each well. Plates (duplicate samples) were made by spreading each sample on Sabouraud glucose agar. The number of cfu was determined after 18 hours of incubation at 37°C. Control cultures consisted of *C. albicans* cells incubated without effector cells.

Phagocytic Activity

The ability of adherent spleen cells to phagocytize heat inactivated *C. albicans* cells was measured by incubating 5×10^6 cells per ml in U-shaped 96-well microtiter plates with 25×10^6 *C. albicans* cells per ml for various times.

After the adherent 0.1 ml of spleen cells were incubated with 0.1 ml of yeast cells, the wells were washed. After that the cells were removed and stained with May-Grunwald Giemsa. The number of intracellular *C. albicans* cells was then determined microscopically by counting the yeast cell content of 100 cells. The counts were done in duplicate by two different observers and the results were averaged.

Statistical Analysis

Differences in survival times were analyzed by the Mann-Whitney U-test. Differences in the number of cfu or specific radiolabel release in the *in vitro* microcytotoxicity assay were determined by the Student's "t" test.

Results

Effect of AmB Administration on *In Vivo* Resistance against *C. albicans* Infection and *In Vitro* Cytotoxic Activity of Spleen Cells

To confirm our previous observations on the AmB-induced increase in resistance to *C. albicans* infection and augmentation of cell-mediated immunity by splenic effectors *in vitro*[1], CD2F1 mice were given a single ip injection of AmB 10 mg/kg at various times before the intravenous challenge with 10^6 *C. albicans* cells. Groups of mice receiving the drug according to the same schedule were used as donors of spleen cells to be reacted *in vitro* against radiolabeled *C. albicans* particles. Table 1 shows the results. It is apparent that AmB treatment considerably enhanced the *in vivo* resistance of mice to microbial challenge even when the latter was performed 6$\sim$8 days after exposure to the drug, at a time when no direct ("chemotherapeutic") effect of AmB could be expected to occur[1]. Table 1 also shows that

Table 1. Effect of *in vivo* administration of AmB on both resistance to *C. albicans* infection and *in vitro* candidacidal activity of spleen cells.

Time of *in vivo* treatment with AmB[a]	Mortality[b]		Spleen cellularity[e] ($\times 10^6$)	^{51}Cr Specific release (%)[f]	
	MST[c]	D/T[d]		100:1	25:1
None	4.5	10/10	80.3	10.9	2.0
1	>60	1/10*	78.5	16.3*	4.9*
2	>60	2/10*	76.6	18.0*	5.3*
4	>60	2/10*	84.5	21.1*	5.5*
6	>60	3/10*	73.7	36.4*	10.6*
8	>60	3/10*	84.1	26.1*	7.0*
12	5.0	10/10	80.2	11.7	3.2

[a] AmB (10 mg/kg) was given as a single ip injection a number of days before microbial challenge or collection of splenocytes for the *in vitro* assay (day 0).
[b] 8-Week old CD2F1 mice were challenged by iv route with 1×10^6 *C. albicans* cells.
[c] MST: Median survival time (days).
[d] D/T: Dead mice at day 60 over total animals tested.
[e] The values reported are the means of individual counts of ten spleens for each experimental group. Standard errors, usually <6%, have been omitted.
[f] Ratios are effector to target cells. Data are the means of quadruplicate samples. Standard errors, usually <1.5%, have been omitted.
* $P<0.01$ according to Mann-Whitney U-test *in vivo* Student's "t" test *in vitro* (AmB-treated *versus* untreated).

Table 2. Effect of separation by adherence on plastic surfaces, nylon wool passage or carbonyl-iron and magnet treatment on candidacidal activity of splenocytes from normal or AmB-treated CD2F1 mice.

Effector cell population	Treatment with AmB[a]	^{51}Cr Specific release (%)		
		10:1[b]	5:1	2.5:1
Untreated cells	−	10.7	5.4	2.0
Plastic nonadherent cells	−	9.0	8.0	7.4
Plastic adherent cells	−	18.6*	9.5*	3.8*
Nylon wool-nonadherent cells	−	0.9*	0.4*	0.2
Carbonyl-iron+magnet-treated cells	−	2.4*	1.3*	0.3
Untreated cells	+	20.6	15.4	8.6
Plastic nonadherent cells	+	5.7*	4.8*	2.3*
Plastic adherent cells	+	36.2*	23.8*	10.3*
Nylon wool-nonadherent cells	+	0.6*	0.3*	0.4*
Carbonyl-iron+magnet-treated cells	+	1.7*	0.5*	0.2*

[a] AmB (10 mg/kg) was given ip as a single injection 8 days before *in vitro* assay.
[b] Ratios are effector to target cells. Data are the means of quadruplicate samples. Standard errors, usually <1.5%, have been omitted.
* $P<0.01$ (fractionated *versus* unfractionated) according to the Student's "t" test.

changes in the candidacidal potential of spleen cells *in vitro* followed a pattern most similar to that observed *in vivo*, with peak levels of reactivity at about 6 days after treatment. One thing to be noted in this experiment is that at no time after AmB exposure did spleen cellularity change, thus suggesting that activation was the major cellular mechanism involved in the *in vivo* effect.

Characterization of Effector Cells, Removal of Adherent and Phagocytic Cells

To characterize the effector cell responsible for cytotoxic activity against *C. albicans* in our system, we studied the effects of removal of plastic or nylon adherent cells from the splenic population of AmB-

Table 3. Effect of treatment with different antisera plus rabbit complement on the candidacidal activity of splenocytes from normal or AmB-treated CD2F1 mice.

Effector cell population	Treatment with AmB[a]	^{51}Cr Specific release (%)[b]		
		10:1	5:1	2.5:1
Untreated cells	—	15.6	9.5	4.7
Complement treated cells	—	14.1	9.6	5.1
Anti-Thy 1.2+complement treated cells	—	15.1	9.8	5.4
Anti-Asialo GM1+complement treated cells	—	15.2	10.3	5.3
Untreated cells	+	28.6	18.8	11.6
Complement treated cells	+	26.7	17.1	10.2
Anti-Thy 1.2+complement treated cells	+	26.4	18.6	9.3
Anti-Asialo GM1+complement treated cells	+	26.1	16.6	8.5

[a] AmB (10 mg/kg) was given ip as a single injection 8 days before *in vitro* assay.
[b] Ratios are effector to target cells. Data are the means of quadruplicate samples. Standard errors, usually <1.5%, have been omitted.

Table 4. Effect of treatment schedule with AmB both on resistance to systemic infection with *C. albicans* and candidacidal activity of spleen cells from CD2F1 mice.

Treatment schedule (days)[a]	Cumulative dose of AmB (mg/kg)	Mortality[b]		^{51}Cr Specific release (%)		
		MST[c]	D/T[d]	10:1[e]	5:1	2.5:1
None	—	5.5	10/10	8.0	6.2	5.2
8	10	>60	3/10*	22.1*	18.9*	10.8*
8	50	>60	1/10*	24.5*	19.9*	12.6*
12, 11, 10, 9, 8	10	>60	4/10*	24.3*	18.3*	15.7*
12, 11, 10, 9, 8	50	>60	0/10*	23.1*	17.4*	10.3*
16, 14, 12, 10, 8	10	5.5	8/10	8.2	5.1	2.5
16, 14, 12, 10, 8	50	4	7/7	9.3	6.4	3.9

[a] AmB was given ip before microbial challenge or collection of splenocytes for the *in vitro* assay (day 0).
[b] 8-Week old CD2F1 mice were challenged by iv route with 1×10^6 *C. albicans* cells.
[c] MST: Median survival time (days).
[d] D/T: Dead mice at day 60 over total animals tested.
[e] Ratios are effector to target cells. Data are the means of quadruplicate samples. Standard errors, usually <1.5%, have been omitted.
* $P<0.01$ according to Mann-Whitney U-test *in vivo* and Student's "t" test *in vitro* (AmB-treated *versus* untreated).

treated animals. Concurrently, the impact of treatment with carbonyl-iron+magnet, which removes phagocytic cells, was also studied. Table 2 shows the results. It is apparent that the cells with candidacidal properties activated by AmB showed an adherence and phagocytic pattern typical of macrophages.

Treatment with Antisera Plus Complement

Various antisera directed against specific cell membrane antigens were used to further characterize the cells responsible for anti-*Candida* cytotoxic activity in our system after exposure to AmB. Table 3 shows that treatment with anti-Thy 1.2 plus complement or anti-Asialo GM1 plus complement, which affect mature T cells and natural killer lymphocytes, respectively, had no major effect on the candidacidal activity of splenic effectors activated by AmB.

Effect of AmB Treatment Schedule

Previous studies have shown that optimal immunostimulatory anti-*Candida* effects follow treat-

Table 5. Candidacidal activity of adherent spleen cells after *in vitro* exposure to different doses of AmB.

In vitro incubation with AmB (μg/ml)[a]	^{51}Cr Specific release (%)[b]		cfu inhibition (%)	
	10:1	5:1	5:1	1:1
Untreated control	14.3	10.4	40.2[d]	21.7
0.1	15.3	11.8	41.8	24.9
0.5	15.8	11.9	57.8	36.1
1.0	17.3	11.4	63.6*	40.3*
5.0	16.3	11.8	72.3*	46.4*
10	28.5*	23.3*	96.1*	51.0*
20	20.3*	16.4*	95.3*	43.0*
10, freeze-thawed[c]	1.2	0.8	3.1	1.2

[a] Plastic adherent spleen cells from CD2F1 mice were cultured for 3 hours with different concentrations of AmB.

[b] Ratios are effector to target cells. Data are the means of quadruplicate samples. Standard errors, usually $<1.5\%$, have been omitted.

[c] Spleen cells treated *in vitro* with AmB 10 μg/ml were disrupted by repeated freeze-thawing prior to their use as effectors in the assay.

[d] The actual number of cfu was 150 in the absence of effectors.

* $P<0.01$ (AmB-treated *versus* untreated cells) according to the Student's "t" test.

ment of mice with a single dose of AmB 10 mg/kg administered 2~8 days before microbial challenge[1]. In the present study, we investigated the impact of repeated drug administrations over a prolonged period of time. Table 4 shows the results. It is apparent that a single dose of 10 mg/kg administered 8 days before *Candida* challenge was as effective as 5 daily (days −12 to −8) exposures to AmB 2 mg/kg. The same fractionated dose, on the other hand, was largely ineffective when administered every other day from day −16 to −8. Similar results were obtained with AmB 50 mg/kg.

In Vitro Activation of Macrophages by AmB

Studies were also conducted in order to establish the optimal conditions of *in vitro* exposure of macrophages which would induce candidacidal activity. Following preliminary indications[1], plastic adherent spleen cells from intact mice were incubated *in vitro* for 3 hours with a range of AmB concentrations (0.1~20 μg/ml). At the end of the incubation time, the cells were extensively washed and used as effectors in a ^{51}Cr-release and a cfu inhibition assay. Table 5 shows the results. It is apparent that strong anti-*Candida* activity was achieved by exposure of macrophages to AmB 10 μg/ml. Freeze-thawed AmB-treated spleen cells had no cytotoxic activity against *Candida*, thus ruling out any direct involvement of AmB which might have remained associated with the effector cells. In subsequent experiments, the effect of incubation with AmB 10 μg/ml for varying lengths of time was studied. Fig. 1 shows that, on exposure of macrophages to the drug for 15, 30, 60, 120 or 240 minutes or 24 hours, optimal anti-*Candida* effects were induced by a contact of at least 60 minutes. A series of experiments was also devoted to clarifying some kinetic aspects of the AmB-induced macrophage activation. Adherent spleen cells, either from intact or AmB-treated (10 mg/kg, day −8) mice were cultured *in vitro* for varying lengths of time before being tested in a ^{51}Cr-release and cfu inhibition assays. Effector cells activated *in vitro* by AmB (10 μg/ml, 3 hours) were also assayed according to the same schedule. Fig. 2 shows that all of the effectors tested gradually lost their cytotoxic potential if cultured *in vitro* in the absence of AmB over a 6-day period.

Kinetics of Phagocytosis by AmB-activated Macrophages

The phagocytic efficiency of macrophages activated by AmB was monitored at different times

Fig. 1. Effect of the time of *in vitro* AmB incubation with normal adherent spleen cells on their candidacidal activity.

○, □, Untreated control spleen cells; ●, ■, spleen cells incubated with AmB.

Plastic adherent spleen cells from normal CD2F1 mice were incubated *in vitro* with medium or AmB (10 μg/ml) for various times. After that the effector cells were repeatedly washed and assayed against *C. albicans* target cells.

Data are the means of quadruplicate samples. Standard errors, usually <1.5% for chromium release assay (CRA) and <4.5% for cfu assay have been omitted. Ratio of effectors to target cells was 10: 1 for CRA and 2.5: 1 for cfu assay.

Arrows, $P<0.01$ (AmB-treated *versus* untreated cells) according to Student's "t" test.

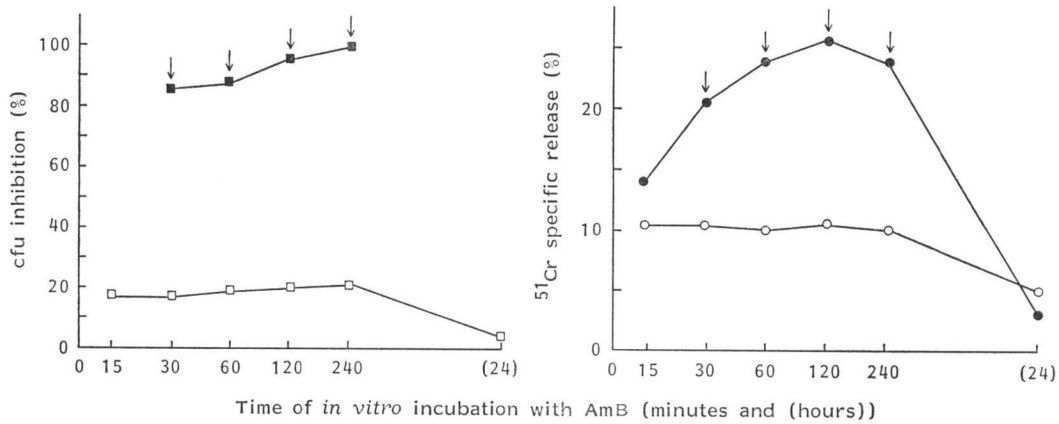


Fig. 2. Persistance *in vitro* of candidacidal activity of plastic adherent spleen cells from normal CD2F1 mice incubated *in vitro* with AmB or from AmB-treated mice.

○, □, Spleen cells from untreated control mice; ●, spleen cells from AmB-treated mice; ■, spleen cells from untreated mice incubated *in vitro* with AmB.

AmB (10 mg/kg) was given ip as a single injection 8 days before collection of splenocytes for the *in vitro* assay. Plastic adherent spleen cells from normal mice were incubated for 2 hours with AmB (10 μg/ml).

Plastic adherent spleen cells from normal or AmB-treated mice or activated *in vitro* with AmB were cultured at different times before their use as effector cells against *C. albicans*.

CRA (A) and cfu (B) assays were performed at effector to target ratio of 10: 1 and 5: 1 respectively. Standard errors, usually <1.5% for CRA and <4.5% for cfu assay, have been omitted.

Arrows, $P<0.01$ (AmB-treated *versus* untreated cells) according to Student's "t" test.

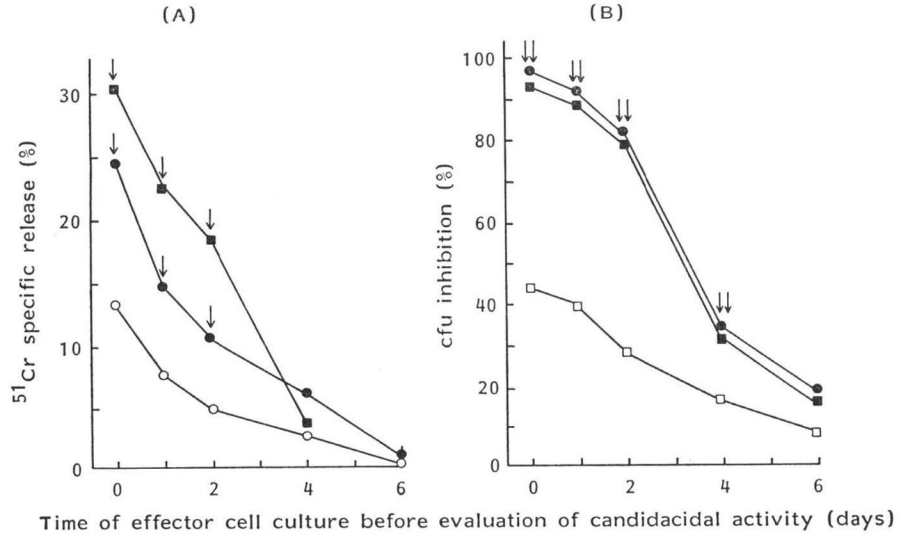
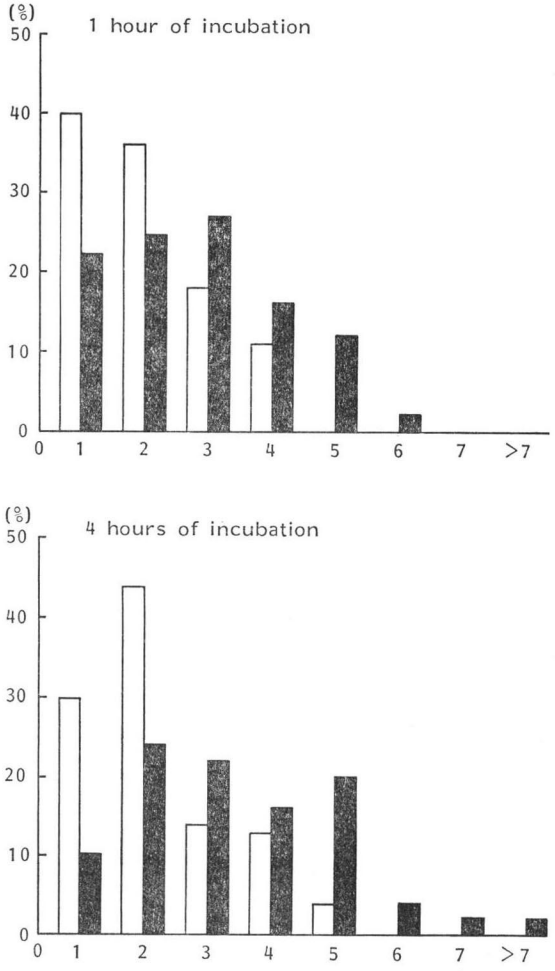


Fig. 3. Effect of AmB administration on phagocytic efficiency *in vitro* of plastic adherent spleen cells against inactivated *C. albicans* microorganisms.

□, Untreated control CD2F1 mice; ■, AmB-treated mice.

The results were obtained on 200 adherent spleen cells.



during the incubation of the effector cells with inactivated *C. albicans* microorganisms. Fig. 3 shows the results in terms of number of yeast particles ingested by a single phagocytic cell at 1 and 4 hours of incubation. It appears that the phagocytic activity of AmB-treated macrophages was considerably higher than that of controls. In another experiment, adherent spleen cells from intact or AmB-treated (10 mg/kg, day −8) mice were incubated *in vitro* with inactivated *C. albicans* at the ratio of 1 : 5. Fig. 4 shows the results in terms of number of ingested yeast particles per 100 effectors as well as percentage of cells that had ingested at least one *C. albicans* particle. Again, the AmB-activated macrophages displayed an enhanced phagocytic ability.

Discussion

The present paper follows previous observations by our group that AmB can exert considerable

anti-*Candida* activity through a mechanism largely independent of its chemotherapeutic properties[1]. Indeed, this activity appeared in our preliminary investigations to be rather related to the immunoadjuvant effects that AmB has been shown to exert in experimental tumor[12] as well as infection models[13,14]. The majority of these studies indicated activated macrophages as the principal mediators of the immunoadjuvant activity of AmB[15~17]. Our present study shows that cells with candidacidal properties can be recovered from mice treated with AmB. These cells appear to be macrophages by both morphological and functional criteria. They are activated *in vivo* by AmB in a dose-dependent fashion under selected treatment conditions. A single dose of 10 mg/kg has effects comparable to those of 5 daily exposures to 2 mg/kg. The activity is lost when the repeated dosages are given on an alternate day basis. No additional benefits, on the other hand, are obtained if the cumulative dose is raised to 50 mg/kg. Thus, macrophage activation by AmB seemed to develop over a prolonged period of time following administration of the drug in adequate amounts. *In vitro* studies of macrophage activation by AmB were performed in which we tested different AmB concentrations and different exposure times. Again there appeared to be a threshold value (10 μg/ml) below which no significant activation could be obtained. Macrophage activation, which was evident in both the chromium release assay (CRA) and cfu inhibition tests, gradually declined when the cells, activated either *in vivo* or *in vitro*, were kept under *in vitro* conditions for varying lengths of time before testing. Studies were also devoted to clarifying the mechanisms by which the activated macrophages exert the increased anti-*Candida* activity. It was found that the phagocytic efficiency of AmB-activated macrophages was considerably enhanced with respect to untreated controls both in terms of kinetic parameters of phagocytosis as well as total numbers of *Candida* particles ingested. Moreover, on incubation of AmB-activated macrophages with live *Candida*, marked inhibition of hyphae formation was observed (data not shown).

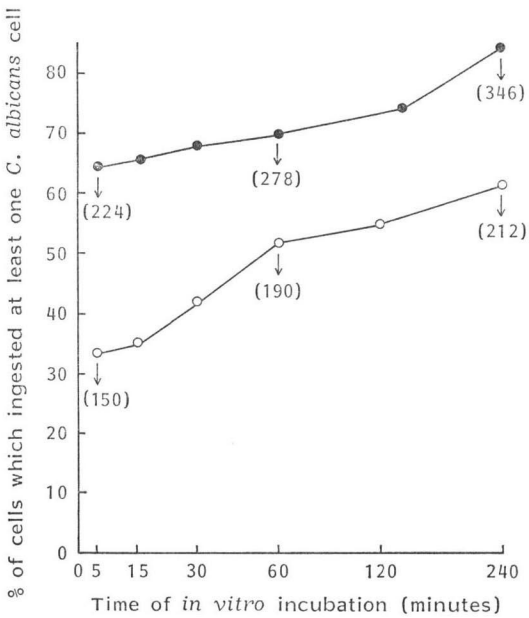
Fig. 4. Effect of AmB administration on *in vitro* phagocytic capability of plastic adherent spleen cells incubated with inactivated *C. albicans* microorganisms.

AmB (10 mg/kg) was given ip as a single injection 8 days before collection of splenocytes for the *in vitro* assay.

Adherent spleen cells from normal (○) or AmB-treated (●) mice were incubated *in vitro* with inactivated *C. albicans* at a ratio of 1: 5.

The results are obtained counting 100 adherent spleen cells for each incubation period.

In parentheses are reported the numbers of phagocyted cells for 100 adherent cells at 5′, 1 or 4 hours of *in vitro* incubation.



In conclusion, the data of the present paper suggests the concept that *in vitro* or *in vivo* exposure of macrophages to AmB results in increased candidacidal activity. This observation might be useful toward a more thorough understanding of the mechanisms underlying the therapeutic efficacy of AmB as an antifungal agent.

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