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Novel Aspect of Amphotericin B Action: Accumulation in Human Monocytes Potentiates Killing of Phagocytosed *Candida albicans*

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The influence of low doses of amphotericin B on the capacity of human monocytes to kill *Candida albicans* was investigated. Killing rates were quantified by a novel flow cytometric assay and were found to be 37% ± 3% (standard error of the mean) after 3 h. Preincubation of monocytes for 6 to 20 h with low concentrations of amphotericin B (0.2 μg/ml) resulted in a markedly augmented fungicidal capacity. Enhancement of killing was 80% ± 11% (standard error of the mean) over that by the controls. This effect did not appear to be due to amphotericin B-dependent monocyte activation; the respiratory burst and expression of human leukocyte antigen-DR were unaltered, and no stimulation of interleukin-1β release occurred. Cell-associated amphotericin B was extracted with acetonitrile and was quantified by scanning spectrophotometry. Amphotericin B appeared to accumulate in the cells, and intracellular concentrations attained after overnight incubation in 1 μg of the drug per ml were estimated to be in the range of 50 fg per cell. The fact that intracellular accumulation was responsible for the enhanced fungicidal capacity of monocytes was supported by the findings that killing of *Staphylococcus aureus* remained normal and enhancement of killing of an amphotericin B-resistant *C. albicans* strain was minimal. Dramatic enhancement of monocyte fungicidal capacity probably extends to other amphotericin B-susceptible fungi and could represent a hitherto unrecognized determinant underlying the curative properties and prophylactic efficacy of this drug.

Despite its toxicity, amphotericin B has remained the prime drug for treating life-threatening fungal infections. This agent binds with high affinity to ergosterol. Its fungicidal action is generally ascribed to an enhancement of membrane permeability as a result of the formation of ion channels (5, 16) and perhaps also because of oxidative damage of membrane constituents (4, 6). Unfortunately, amphotericin B also binds to cholesterol in mammalian cell membranes and elicits severe toxic effects. Many efforts have therefore been devoted to establishing treatment regimens that combine the merits of antifungal activity with a minimum of untoward side effects. The possibilities of local amphotericin B application are also receiving increased attention. Aerosol application, first described nearly 20 years ago (23), and low-dose intravenous application are emerging as prophylactic measures against pulmonary fungal infections in neutropenic patients (13, 20, 29).

General belief holds that the beneficial effect of amphotericin B derives mainly from its direct fungicidal action. In addition, the drug reportedly can act on macrophages to stimulate [^{3}H]thymidine uptake (5) and the respiratory burst (34, 35), promote tumor necrosis factor secretion (9), and enhance the tumoricidal (8, 26) and microbicidal (3, 24, 26, 32) actions of these cells. The mechanisms underlying these processes and the relevance thereof remain unclear.

We have been analyzing the effects of cytokines and antifungal agents on the phagocytic function of human monocytes. These studies have been facilitated by the use of novel flow cytometric assays that permit rapid analyses to be performed with a high degree of accuracy (18, 19). The assays were first introduced for quantifying granulocyte phagocytic function and have now been adapted to the analysis of monocytes. In the studies described here, we showed that monocytes preincubated for hours with low doses of amphotericin B acquire a remarkably augmented capacity to kill ingested *Candida albicans*. This effect appears to be due to the intracellular accumulation of the drug rather than to a nonspecific stimulation of the cells. Potentiation of the fungicidal capacity of monocytes is likely to be an important novel determinant underlying the therapeutic efficacy of amphotericin B.

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MATERIALS AND METHODS

Microorganisms. An isolate of *C. albicans* from our diagnostic laboratory or an amphotericin B-resistant mutant, kindly provided by H. Dermoumi, Essen, Germany, was cultured at 37°C in tryptic soy broth for 16 to 20 h. A total of 1.5 ml of yeast cell cultures was centrifuged and the pelleted cells were resuspended in 1 ml of saline and incubated with 2 μM bis-carboxyethyl-carboxyfluorescein pentaacetoxymethylester (BCECF-AM; Becton Dickinson, Heidelberg, Germany) for 30 min in an Eppendorf Thermomixer 5436 (Eppendorf Inc., Hamburg, Germany) at 37°C and 1,100 rpm. Following three washes with saline, yeast cells were resuspended in saline and the mixture was kept on ice. Cells were counted in a Neubauer counting chamber.

In some experiments *Staphylococcus aureus* Wood 46 was used as the target. An overnight bacterial culture was spun down, washed three times, resuspended in saline, and kept on ice until use. The density of bacteria was determined photometrically (550 nm).

Preparation of monocytes. Monocytes were isolated from

buffy coats as described by Denholm and Wolber (14), with some modifications. Buffy coats were diluted 1:1 with phosphate-buffered saline (PBS), applied to Ficoll (3 volumes of blood, 2 volumes of Ficoll), and centrifuged (500 × *g*, 30 min, 25°C). Mononuclear cells were washed once in PBS and resuspended in 8 ml of PBS. A total of 4 ml of the cell suspension was gently mixed with 8 ml of Percoll suspension. This suspension was prepared by mixing 10 ml of Percoll (Sigma, Munich, Germany) with 1.65 ml of 10-fold-concentrated Hanks balanced salt solution (HBSS). The pH was adjusted to 7.0 with 1 M HCl. After centrifugation for 30 min at 480 × *g* (25°C; 2,000 rpm rotor; SS 34 Sorvall centrifuge) the top layer (~400 μl) was retrieved and the cells were washed in 50 ml of PBS (for 5 min, as described above). The final pellet was resuspended in RPMI (without glutamine; Seromed; Biochrom, Berlin, Germany) with 10% serum (normal human serum, blood group AB). The cells were counted, and the percentage of monocytes was determined by flow cytometry. Monocytes were differentiated from lymphocytes according to their relative cell volumes and granularities. Usually, monocytes accounted for over 90% of the cells. They were diluted in RPMI–10% serum to 1.2×10^6 monocytes per ml, and 125 or 500 μl was applied to each well of 96- or 24-well culture plates, respectively (flat-bottom culture plates; Greiner, Solingen, Germany). The plates were centrifuged at 500 × *g* for 10 min at 25°C to sediment the cells. Unless otherwise stated, amphotericin B or stimuli were then added and the cells were incubated at 37°C with 5% CO_2.

Preparation of human alveolar macrophages. Bronchoalveolar fluid obtained from lung cancer patients for diagnostic purposes was filtered through gauze and was collected in 50-ml Greiner tubes. After centrifugation (500 × *g*, 10 min, 4°C), contaminating erythrocytes were lysed with 3 ml of ice-cold distilled water (30 s; and then with 1 ml of 0.6 M KCl). Cells were washed once in PBS, adjusted to 900,000 macrophages per ml of RPMI–10% serum, and seeded onto 96-well culture plates (125 μl per well).

Drugs and reagents. Amphotericin B (Squibb Pharma, Vienna, Austria) was dissolved in distilled water, as provided by the manufacturer. The stock solution (5,000 μg/ml) was kept at −20°C. Recombinant human gamma interferon (IFN-γ; Gammaferon 50; Bioferon GmbH & Co., Laupheim, Germany) was diluted 1:100 in PBS with 0.1% human serum albumin (Behring, Marburg, Germany) to yield a stock solution of 500 μg/ml, and the solution was kept at −70°C. Lipopolysaccharide (100 μg/ml in RPMI) from *Escherichia coli* O55:B5 (Sigma) was kept at −20°C.

Yeast killing assay. The yeast killing assay method was based on the flow cytometric assay for quantifying killing of *C. albicans* by neutrophils (18). Viable yeast cells exhibit green fluorescence (fluorescence 1 [FL 1]) because of intracellularly trapped BCECF. Nonviable cells lose their fluorescence after treatment with sodium deoxycholate. The ratio of viable, green fluorescent yeasts to dead, nonfluorescent yeasts was quantified by flow cytometry.

Methodology for the yeast killing assay. After overnight incubation, the supernatants of monocytes were carefully removed and 125 μl of fresh RPMI–10% serum, supplemented with 0.1 μg of amphotericin B per ml and 300,000 yeast cells per ml, was added, yielding a ratio of effector to target cells of 4:1. Amphotericin B at this concentration was not fungicidal but prevented pseudohyphae formation and aggregation of yeast cells, which otherwise generated artifacts in the killing assays. The plates were centrifuged (500 × *g*, 10 min, 4°C) to sediment the yeast cells and were incubated at 37°C. After the desired period, 125 μl of sodium deoxycholate (25 mM in distilled water) was added to each well. Following an incubation of 3 min at room temperature, 200 μl was transferred to Eppendorf tubes containing 500 μl of water for 3 min at 37°C (Thermomixer). Finally, 200 μl of saline was added and the samples were kept on ice until analysis. Yeast cells incubated in parallel without monocytes served as controls. Flow cytometry was performed with a FACScan flow cytometer and Lysis II software (Becton Dickinson, Heidelberg, Germany). Forward scatter (FSC) and sideward scatter (SSC) signals were amplified logarithmically. The SSC threshold ranged between channels 300 and 360. A live gate was set around yeast particles, defined by their relative cell volumes (FSC) and relative granularities (SSC) (Fig. 1A). The relative numbers of dead yeasts were reflected by the increase in the nonfluorescent yeasts (Fig. 1B to G). The validity of the assay has previously been rigorously tested by parallel determinations of CFU (18). In the present work, similar controls were included in several sets of experiments, and a total of 12 parallel determinations were performed. The proportion of yeast killing by colony countings was always within ±15% of the values obtained by flow cytometry. In particular, it is stressed that the enhancement of killing of *C. albicans* by monocytes preincubated with amphotericin B was confirmed by plating out and colony countings in four different experiments, and these results did not deviate from the flow cytometric quantitations.

Killing of *S. aureus*. After overnight incubation, supernatants of monocyte cell cultures were removed and RPMI with 10% serum containing *S. aureus* was added. The target-to-effector cell ratio was 1:1, 5:1, or 10:1. After centrifugation and incubation at 37°C for 0.5, 1, or 2 h, monocytes were lysed with 125 μl of an aqueous 1% 3[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS; Sigma) solution for 3 min at room temperature. Then, 200 μl was transferred to Eppendorf tubes with 800 μl of water, and the mixture was incubated at 37°C for another 3 min. After sonication (10 s; constant output at lowest level; Branson Sonifier 250), the samples were appropriately diluted and plated out for determination of CFU. As controls, bacteria were incubated without monocytes. Sonication was essential in order to disperse the staphylococci, which formed clusters during incubation without cells.

Determination of oxidative burst. Respiratory burst was detected by flow cytometry by the method published by Rothe et al. (28) for granulocytes. Dihydrorhodamin 123 (DHR) freely penetrates into cells and is oxidized by H_2O_2 to generate a green fluorescent product (rhodamine 123). The percentage of green fluorescent cells and their relative fluorescence intensities serve as parameters for the respiratory burst (H_2O_2 formation). These measurements do not yield absolute values for the H_2O_2 generated but allow direct comparison of the respiratory burst at a single-cell level. Thus, increases or decreases relative to the controls are seen as increases or decreases in fluorescence of the individual particles (28). Two sets of confirmatory experiments were also conducted by using continuous recordings of lucigenin chemiluminescence (2a), with the same results.

Methodology for determination of oxidative burst. Supernatants of overnight monocyte cultures were discarded and adherent monocytes were detached by immersion in 1 mM EDTA–136.8 mM sodium chloride–2.6 mM potassium chloride–8.1 mM Na_2HPO_4–1.4 mM KH_2PO_4–1.1 mM glucose (pH 7.2). After 10 min on ice, cells were washed once with RPMI–2% serum. The pellet was resuspended in RPMI–10% serum, and the cells were counted. Recovery of monocytes was nearly complete. Monocytes were diluted to 1.2 ×

10^6/ml, and aliquots of 200 μl were prepared in Eppendorf tubes. Either unlabelled yeasts at different ratios of effector to target (4:1, 1:1, 1:3) cells, fMLP or phorbol myristate acetate (PMA; 100 nM; Sigma), or PBS (as control) was added. Aliquots of 50 μl were incubated at 37°C in the Thermomixer. At selected time points, 1 ml of ice-cold HBSS without calcium or magnesium was added, and all samples were collected on ice. The 0-min value was directly pipetted into HBSS. Finally, 10 μl of 100 μM DHR (Becton Dickinson, Heidelberg, Germany) was added, and the samples were incubated for 15 min at 37°C in the Thermomixer. Samples were kept on ice for a maximum of 2 h prior to flow cytometric measurement.

Staining for HLA-DR. Monocytes detached from culture plates were suspended in 500 μl of RPMI–2% serum and were stained with IOT 2a (monoclonal antibody against human leukocyte antigen [HLA]-DR, fluorescein isothiocyanate [FITC] labelled; Dianova, Hamburg, Germany) or an appropriate isotype control (Dako Diagnostica, Hamburg, Germany) for 20 min on ice. After one wash with RPMI–2% serum, cells were resuspended in 500 μl of 0.1% paraformaldehyde in PBS and were analyzed by flow cytometry. Amplification of FSC and SSC was linear; the FSC threshold was set at 52. Five thousand monocytes were analyzed for their green fluorescence, reflecting relative numbers of surface HLA-DR molecules.

Quantification of IL-1β. The interleukin-1β (IL-1β) contained in overnight culture supernatants was determined by an enzyme-linked immunosorbent assay (ELISA; Medgenix Diagnostics, Ratingen, Germany, or Eurogenetics, Eschborn, Germany).

Quantification of cell-associated amphotericin B. Amphotericin B was quantified by scanning spectrophotometry as described by Shihabi et al. (31). Monocytes (2×10^6/ml) in RPMI–10% serum were cultured in six-well flat-bottom culture plates (4 ml per well; Costar; Tecnomara, Fernwald, Germany). After overnight incubation, the culture supernatants were discarded; cells and were washed twice in PBS–400 μl of acetonitrile (for chromatography; Merck, Darmstadt, Germany) were added in order to extract the amphotericin B. After mixing vigorously for 30 s, the samples were pipetted into Eppendorf tubes and the tubes were centrifuged (Eppendorf table centrifuge). Two hundred microliters of supernatant was transferred into quartz microcuvettes (Zeiss, Frankfurt, Germany). Scanning photometry was performed from 350 to 450 nm with computer assistance (Ultrospec II, wavelength scanning; Pharmacia LKB Biotechnology, Cambridge, England), and the difference in the absorbance units between the trough at 398 nm and the peak at 408 nm was determined. The amphotericin B concentrations were calculated on the basis of an amphotericin B standard curve obtained after linear regression analysis.

Control experiments were also performed in order to exclude the possibility that amphotericin B simply adhered to the plastic wells, thus creating artifacts. For this, the drug was applied to empty wells (without cells), and the acetonitrile extraction procedure was performed after overnight incubation as described above. In these cases, no amphotericin B could be detected in the extracts, thus virtually excluding possibilities of an artifact.

RESULTS

Killing of *C. albicans* by monocytes quantified by flow cytometry. Monocyte monolayers cultured in microtiter plates were seeded with labelled *C. albicans* at a relatively high effector-to-target cell ratio. This was essential in order to guarantee that over 90% of the yeast cells would be phagocytosed within 30 min as confirmed by flow cytometric quantification of unassociated yeasts (data not shown). After 1, 3, and 5 h, cells were lysed in the microtiter plates by adding deoxycholate. This step liberated the yeast cells, which fell into two populations: viable and fluorescent or dead and nonfluorescent. The two populations were quantifiable by flow cytometry. In pilot experiments, the results were verified by plating out and colony counting. Further control countings were performed throughout, and the results of the flow cytometric method versus those of the colony countings were always within ±15% of each other. Figure 1 depicts the principle of this method. The results of 24 experiments are shown in Fig. 2. Using this method, we determined that monocytes from healthy volunteers killed 37% ± 3% (standard error of the mean [SEM]) of ingested *C. albicans* within 3 h. Killing did not increase after an additional 2 h of incubation. The value at 3 h was compatible with earlier data of Lehrer (17).

Toxicity of amphotericin B toward *C. albicans* in the presence of 10% normal human serum. As reported previously, amphotericin B at a concentration of approximately 0.5 μg/ml was subfungicidal, inhibited pseudohyphae formation, and suppressed yeast cell aggregation in the presence of 50% heparinized plasma (18, 22). This concentration is greater than the MIC for susceptible *C. albicans*, because the fungicidal action of amphotericin B is markedly reduced in the presence of serum (1, 11, 15). Thus, amphotericin B concentrations of <0.5 μg/ml are devoid of candidacidal effects in human serum, and this has been reported in detail previously (18, 22). In the present study, yeasts were incubated with monocytes in the presence of 10% normal human serum, and we reexamined the action of amphotericin B under this condition. We found a concentration of 0.1 μg/ml to be approximately equivalent to 0.5 μg/ml in 50% plasma; i.e., this concentration was not fungicidal but inhibited pseudohyphae formation and cell aggregation. Concentrations exceeding 0.1 μg/ml were only slightly fungicidal, and killing of 10 to 25% of the yeasts occurred within 3 h.

Preincubation of monocytes with amphotericin B augments subsequent candidacidal capacity. Monocytes were incubated with increasing concentrations of amphotericin B (0 to 0.2 μg/ml) in the presence of 10% normal human serum overnight at 37°C. Thereafter, the medium was removed and replaced with fresh medium containing 0.1 μg of amphotericin B per ml and yeast cells. Amphotericin B was applied to prevent germ tube formation by those few yeast cells that had escaped ingestion. In some experiments that were terminated after 1 h (a period too short to allow germ tube formation), amphotericin B was omitted without changes in the results. Usually, killing of *C. albicans* was assessed after 3 h. As shown in Fig. 3, preincubation with amphotericin B augmented the capacity of monocytes to kill ingested *C. albicans* in a dose-dependent fashion. This effect commenced at amphotericin B concentrations of approximately 0.05 μg/ml and was very prominent at 0.2 μg/ml. The candidacidal capacity increased by 80% ± 11% (SEM), with a maximum of 170% compared with that for the controls. In absolute numbers, this meant that killing rates rose from 37% ± 3% (SEM) (Fig. 2) to 65 to 85%; i.e., in some experiments, killing rates in amphotericin B-pretreated cells were almost three times as high as those in the controls.

The effect of the duration of preincubation was studied. In these experiments, monocytes were incubated with 0.2 μg of amphotericin B per ml for 0 to 20 h and were then challenged

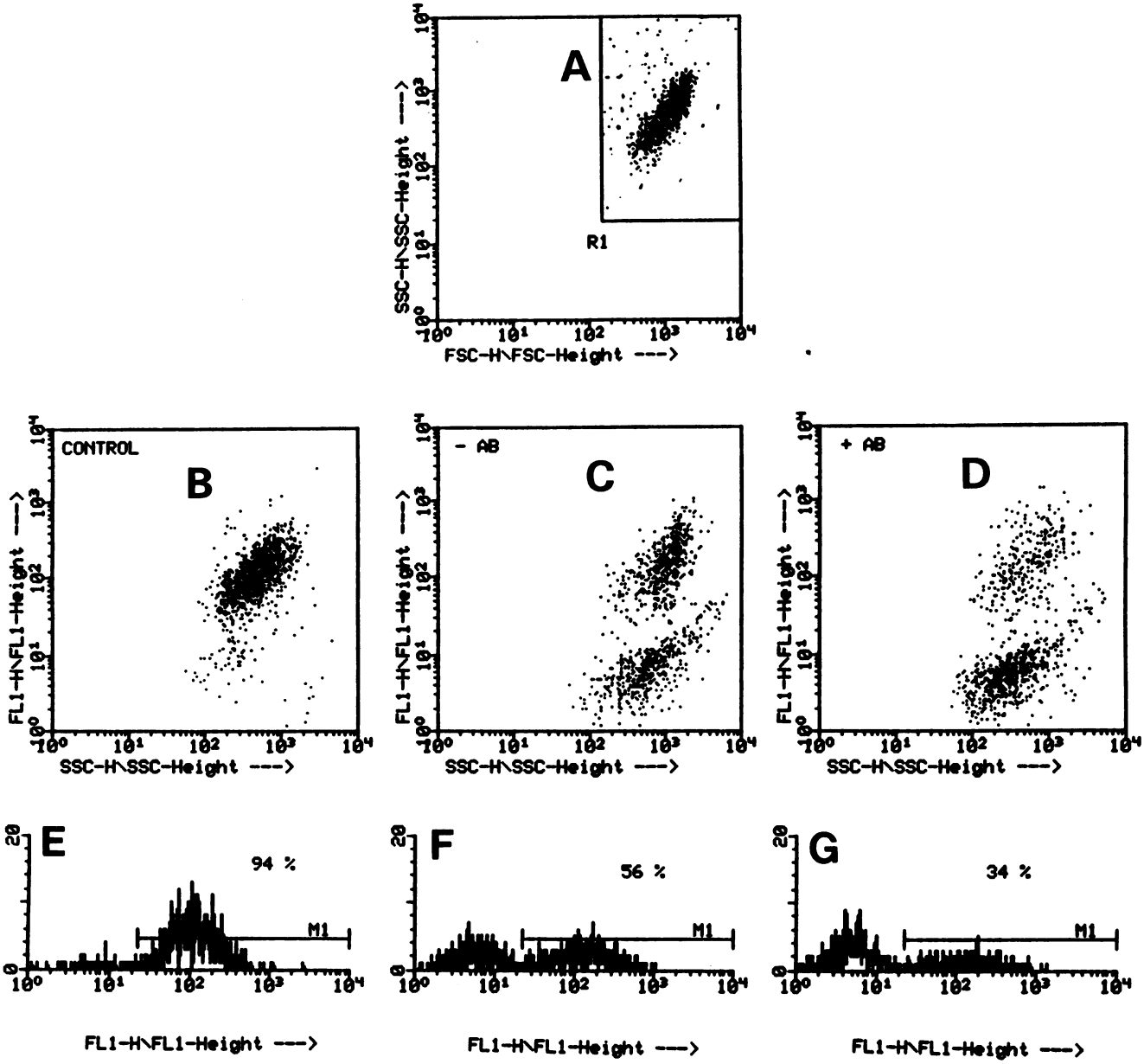

FIG. 1. Principle of flow cytometric assay for killing of *C. albicans*. (A) Yeasts as they present according to their relative cell volume (FSC) and relative granularity (SSC) after detergent treatment. Particles outside region 1 (R1) were excluded from data acquisition. (B to D) Yeasts are depicted according to their relative granularities and relative green fluorescences (FL 1) arising from vital staining with BCECF. (B) Control yeasts incubated in medium without cells; (C) yeasts incubated with cells without amphotericin B pretreatment; (D) yeasts incubated with cells that had been preincubated with 0.1 μg of amphotericin B per ml overnight. (E to G) Quantitative depiction of the distribution of fluorescent (viable) versus nonfluorescent (nonviable) yeast particles; the percentage of fluorescent particles is given in each case. In the control (A; medium without cells), nearly all yeast cells (94%) were viable. In the presence of monocytes, yeasts were killed and lost their green fluorescence (B). Killing was augmented after preincubation with 0.1 μg of amphotericin B per ml (C; with amphotericin B, 34% viable yeasts), compared with that by control monocytes (B; without amphotericin B, 56% viable yeasts).

with *C. albicans* in the presence of 0.1 μg of amphotericin B per ml. As shown in Fig. 4, augmented killing became detectable after a preincubation period of approximately 4 h, and clear effects were noted after 6 to 8 h. These findings underlined the necessity for prolonged incubation of monocytes with amphotericin B in order for killing enhancement to occur. They show that the presence of external amphotericin B alone does not induce the enhanced killing effect.

In two experiments, monocytes were preincubated overnight with 0.2, 0.5, and 1 μg of amphotericin B per ml. The mean increases in candidacidal activity were 96, 136, and 161%, respectively, compared with that for the controls.

Preincubation for 6 h at 4°C also enhanced the killing capacity of monocytes, but to a lesser extent. In three experiments, enhancement of yeast killing by monocytes preincubated with the drug for 6 h at 37°C was 55, 70, and

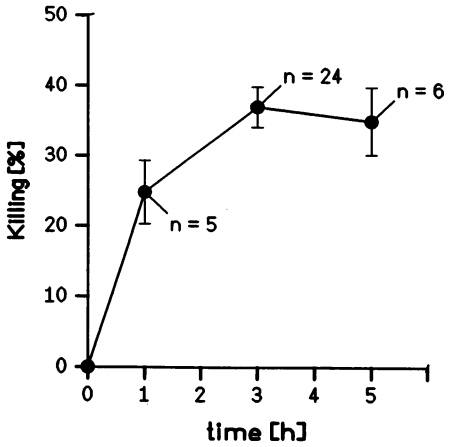

FIG. 2. Kinetics of killing of *C. albicans* by monocytes after overnight culture. The effector-to-target cell ratio was 4:1. The number of experiments for each determination is given. Values are expressed as means ± SEMs.

75%, respectively, over that by the controls, whereas enhancement of killing by monocytes pretreated with the drug at 4°C was only 28, 33, and 29%, respectively, over that by the controls.

In the absence of monocytes, 0.1 μg of amphotericin B per ml was entirely devoid of fungicidal activity in the presence of 10% serum. When yeasts were treated with 0.2 μg of the drug per ml for 3 h, some killing because of the drug alone did occur and ranged from 10 to 25% in different experiments. This should be compared with killing rates of 60 to 85% in the presence of monocytes (Fig. 3).

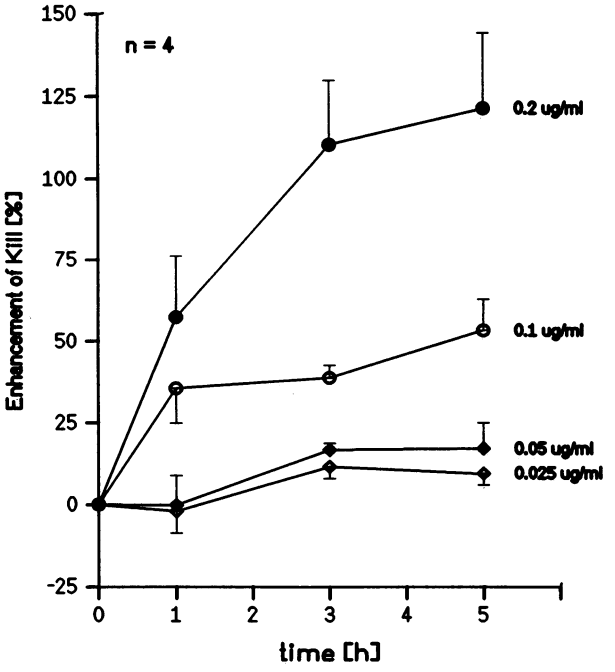

FIG. 3. Enhancement of fungicidal capacity of monocytes following overnight incubation with amphotericin B at the indicated concentrations. Monocytes were incubated with *C. albicans* at an effector-to-target cell ratio of 4:1. Enhancement of killing compared with that by controls commenced at an amphotericin B dose of 0.05 μg/ml. At 0.1 or 0.2 μg/ml, enhancement of killing by amphotericin B was already observed after 1 h (35% ± 10% or 57% ± 19%, respectively) and was very prominent after 5 h (53% ± 10% or 122% ± 23%). The values are means ± SEMs of four experiments.

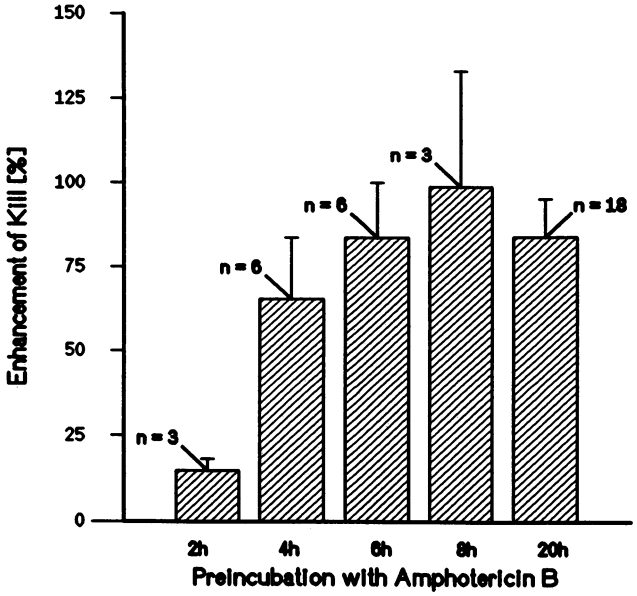

FIG. 4. Dependency of killing enhancement on duration of incubation with amphotericin B. Monocytes were cultured without or with amphotericin B (0.2 μg/ml) for 2, 4, 6, 8, or 20 h. Killing of *C. albicans* was determined after 3 h of incubation; the effector-to-target cell ratio was 4:1. Enhancement of killing was clearly detectable after 4 h of incubation. Data are expressed as means ± SEMs.

Preincubation with amphotericin B enhances candidacidal activity of human alveolar macrophages. The effect of overnight preincubation of human alveolar macrophages (n = 3) with 0.2 μg of amphotericin B per ml was tested. Killing of *C. albicans* after 3 h of incubation was 31% ± 10% (SEM). In the presence of amphotericin B, enhancement of killing was 52% ± 3% (SEM).

Amphotericin B-induced enhancement of candidacidal activity is not associated with characteristic signs of monocyte activation. The experiments described below were performed to obtain an indication of whether amphotericin B-dependent augmentation of candidacidal activity in monocytes was due to monocyte activation. First, the respiratory burst was assessed by flow cytometry. After an overnight incubation in the presence or absence of 0.2 μg of amphotericin B per ml, monocytes were detached from microtiter plates and were incubated with *C. albicans*. The respiratory burst was assessed by measurement of the green fluorescence emitted by the oxidized dye rhodamine 123. Figure 5 depicts the results of an experiment in which two findings were noteworthy. First, on a numerical basis, the populations of monocytes exhibiting green fluorescence were approximately equivalent, irrespective of whether monocytes were treated with amphotericin B. By fluorescence microscopy, we confirmed that only phagocytes that ingested yeasts exhibited green fluorescence. Thus, the relative percentage of cells that became engaged in the phagocytic process was not recognizably influenced by amphotericin B pretreatment. Second, the degree of fluorescence was also equivalent in both cell populations (treated or not treated with amphotericin B), and there was no indication that pretreatment with amphotericin B stimulated or augmented

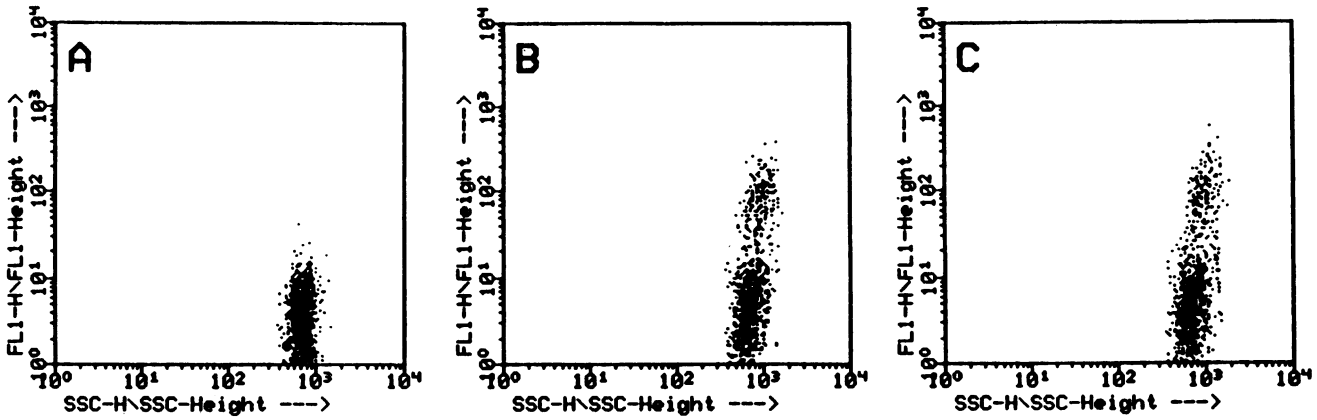

FIG. 5. Example of flow cytometric detection of oxidative burst. Monocytes were acquired at logarithmic amplification of FSC (relative cell volume) and SSC (relative cell granularity) signals and are depicted according to their relative granularities and green fluorescences (FL 1). The latter is generated through oxidation of DHR to rhodamine 123 by H_2O_2. (A) Monocytes after 30 min of incubation without any stimuli. All cells remained nonfluorescent. (B) Monocytes after 30 min of incubation with *C. albicans* at an effector-to-target cell ratio of 1:3. A total of 33% of the monocytes exhibited green fluorescence because of the marked activation of respiratory burst during phagocytosis. (C) Same as panel B, but the monocytes were preincubated overnight with 0.2 μg of amphotericin B per ml; 28% of the cells exhibited green fluorescence.

the subsequent respiratory burst that occurred during phagocytosis. When fMLP or PMA was used as a stimulus, all cells assumed a green fluorescence, reflecting activation of the respiratory burst, and no difference between control monocytes and amphotericin B-treated monocytes was observed (data not shown).

Second, we examined whether amphotericin B treatment induced enhanced expression of HLA-DR, characteristic of immunologically activated antigen-presenting cells. Monocytes were stained with an FITC-labelled monoclonal antibody directed against HLA-DR. IFN-γ-treated cells were taken as positive controls. As shown in Fig. 6, there was enhancement of HLA-DR expression in IFN-γ-treated monocytes, but not in cells pretreated with amphotericin B. In four independent experiments, the mean values for green fluorescence, reflecting the numbers of HLA-DR molecules on the cell surface, did not differ between control cells and cells pretreated with amphotericin B. Cells stained with unspecific FITC-labelled control antibody (isotype control) displayed no fluorescence in any case (one control shown in Fig. 6A; the other controls were identical).

Third, we tested whether amphotericin B induced production of IL-1β. Cells were incubated for 16 h with 0.2 μg of amphotericin B per ml, 1 μg of lipopolysaccharide per ml, or medium. The IL-1β in the supernatants was quantified by ELISA. In three experiments, the levels of IL-1β found after lipopolysaccharide stimulation were 10 to 15 ng/ml, whereas levels detected after amphotericin B treatment were less than 0.7 ng/ml, which was similar to the values for controls, which ranged from 0.18 to 0.5 ng/ml in the different experiments.

Determination of cell-associated amphotericin B. The results presented above indicated that the enhanced candidacidal capacity of monocytes was not due to activation by amphotericin B. To test the possibility that the drug accumulated intracellularly, amphotericin B was extracted from monocytes with acetonitrile and the concentrations were determined by scanning spectrophotometry. As originally reported by Shihabi et al. (31), amphotericin B extracted into acetonitrile exhibits three peaks of absorbance at 366, 384, and 408 nm (Fig. 7A). The authors established a standard curve on the basis of the peak height at 408 nm. In our experiments, the relative height of the third absorbance peak, measured from the trough at 398 nm between peaks 2 and 3, exhibited a linear relationship with the amphotericin B concentration (range, 0.2 to 5 μg/ml). Extraction of washed monocytes with acetonitrile yielded zero background, so that direct determination of cell-associated drug concentrations became feasible. The only prerequisite for this was the use of large numbers of cells and extraction with a small volume of acetonitrile. We also spiked extracts of control cells with known amounts of amphotericin B and found the resulting spectrograms to be equivalent to those for the serum controls.

Monocytes were incubated with 0.5 and 1 μg of amphotericin B per ml. These higher concentrations were applied because the amounts of the cell-associated drug otherwise lay at the threshold of detectability. As shown in Fig. 7B, the cell-associated amphotericin B concentration became detectable by this assay. Kinetic studies performed with 1 μg of amphotericin B per ml indicated that the drug bound rapidly to the cells, and binding was already observed 30 min after incubation. Cell-associated levels of the drug increased over time, with maximal values obtained after 20 h (Fig. 7C). We estimated that overnight incubation with 1 μg of amphotericin B per ml resulted in intracellular levels of 50 fg of the drug per cell (Fig. 7C). To discern whether uptake of amphotericin B was dependent on membrane traffic, cells were incubated with 1 μg of the drug per ml at 4°C overnight, washed, and extracted with acetonitrile. Levels of cell-associated amphotericin B comparable to those obtained after 20 h of incubation at 37°C were noted. However, killing enhancement was always lower (in the range of 50%) than that observed when cells were incubated with amphotericin B at 37°C. This finding could be taken as indirect evidence that amphotericin B first absorbs to the plasma membrane and is then transferred to intracellular compartments via membrane traffic, where it could exert its fungicidal activity. Radioactive amphotericin B preparations are unfortunately not available, and the question regarding the fate of cell-associated drug can therefore not be answered with any certainty at present.

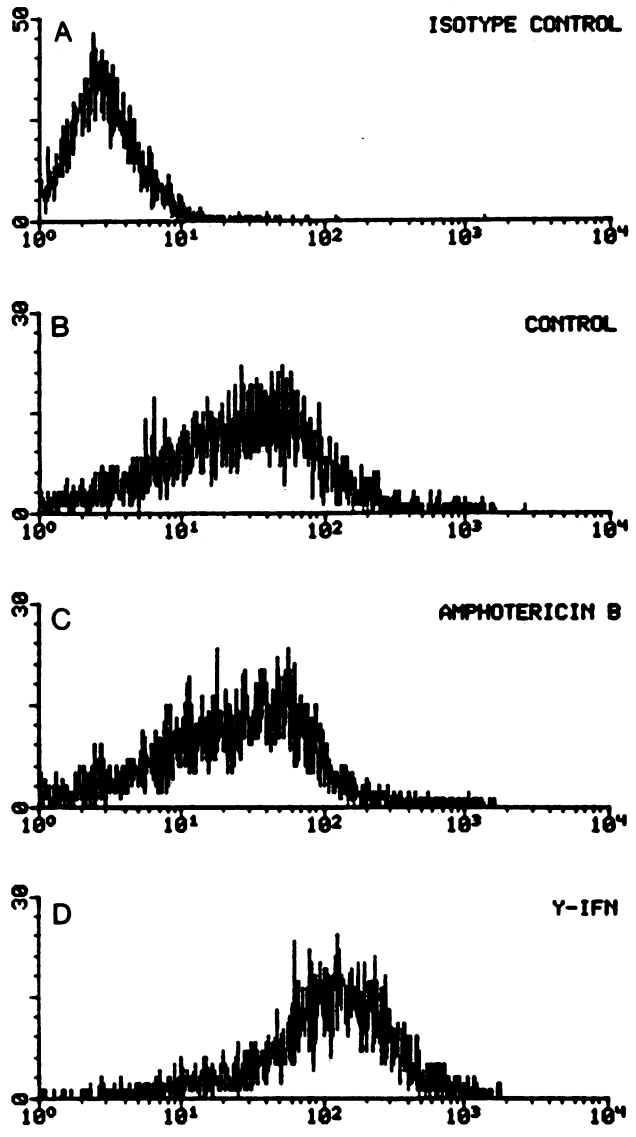

FIG. 6. HLA-DR expression in monocytes pretreated with amphotericin B or IFN-γ. After overnight culture with or without amphotericin B (0.2 μg/ml) or IFN-γ (5 ng/ml; 100 U/ml), monocytes were stained with FITC-labelled monoclonal antibody against HLA-DR or with an unspecific FITC-labelled control antibody (isotype control). Five thousand cells were analyzed for their relative green fluorescences (FL 1). Preincubation with amphotericin B did not upregulate HLA-DR molecules. In contrast, upregulation was readily demonstrable in IFN-γ-treated cells.

Further results that corroborated the contention that the enhanced fungicidal capacity observed as described above was due to accumulation of cell-associated amphotericin B were obtained. First, experiments were performed with *S. aureus* organisms as target organisms. In this case, however, no enhanced killing was observed. Killing of *S. aureus* was approximately 70, 65, or 55% after 60 min in control as well as amphotericin B-treated monocytes at ratios of monocytes to bacteria of 1:1, 1:5, and 1:10, respectively.

Second, an amphotericin B-resistant strain of *C. albicans* was used. The amphotericin B MIC for this strain was 4 μg/ml (determined by H. Dermoumi). The MICs for amphotericin B-susceptible strains were 0.2 to 0.8 μg/ml (30). Monocytes were incubated with 0.2, 0.5, and 1 μg of amphotericin B per ml overnight. Whereas enhancement of killing of the standard *C. albicans* strain was increased by 96% ± 4% (SEM) with a 0.2-μg/ml amphotericin B pretreatment, enhancements of killing of strain R64 were only 26% ± 5%, 54% ± 8%, and 76% ± 26% at the three concentrations given above, respectively (n = 3; ± SEM). These results were consistent with the notion that enhanced killing is due to accumulation of amphotericin B within the cells.

DISCUSSION

The results obtained in the present investigation document a novel finding that may be relevant to amphotericin B therapeutic strategies. The capacity of human monocytes and alveolar macrophages to kill ingested *C. albicans* is augmented by preincubation with amphotericin B. Our results indicate that this is due primarily to accumulation of the drug within the cells. We used a spectrophotometric method to quantify cell-associated amphotericin B. This method was developed by Shihabi et al. (31) for measuring concentrations of the drug in serum. Our findings presented here indicate that it can easily be used for determining intracellular levels as well. The method is far easier to perform than high-pressure liquid chromatographic analysis.

Accumulation of amphotericin B in monocytes did not detectably exert toxic effects on the cells. The cells did not appear to be activated, as assessed by a number of independent criteria. Neither oxidative burst, IL-1β secretion, nor HLA-DR expression was enhanced after preincubation with amphotericin B. Enhanced oxidative burst of human macrophages after preincubation with amphotericin B, as described by Wilson et al. (34), required higher amphotericin B concentrations than the ones that we used. The fact that the intracellular accumulation of amphotericin B was responsible for the enhanced fungicidal effects was supported by the findings that killing of *S. aureus* was normal and killing of an amphotericin B-resistant *C. albicans* strain was only weakly enhanced.

Two quantitative aspects of the present work are noteworthy. First and foremost, the degree of killing enhancement was remarkable. Three-hour killing rates increased from 30 to 40% to 65 to 85%, i.e., by approximately 80 to 100%. By comparison, augmentation of killing induced by IFN-γ is far lower. As to be reported in another communication, IFN-γ enhanced killing by only ~25% compared with that by the controls. The effects of other cytokines, including granulocyte macrophage–colony-stimulating factor are also minimal (unpublished data).

The second quantitative aspect relates to the relatively low concentrations of amphotericin B required to elicit the observed effect. During systemic administration, levels of 1 to 4 μg/ml in plasma (1, 2, 7, 11, 15) result in levels of 27 to 43 μg/g (liver), 9 to 22 μg/g (spleen), and 4 to 10 μg/g (kidney) in various tissues (10, 12, 15). In our experiments, we estimated that the amounts of cell-associated amphotericin B were in the range of 30 to 50 fg per cell after preincubation with 1 μg of the drug per ml. This would correspond to an intracellular concentration of 15 to 30 μg/ml (assuming a cell volume of 500 fl per cell). Thus, the estimated concentrations of cell-associated amphotericin B in our studies are comparable to the concentrations actually measured in the

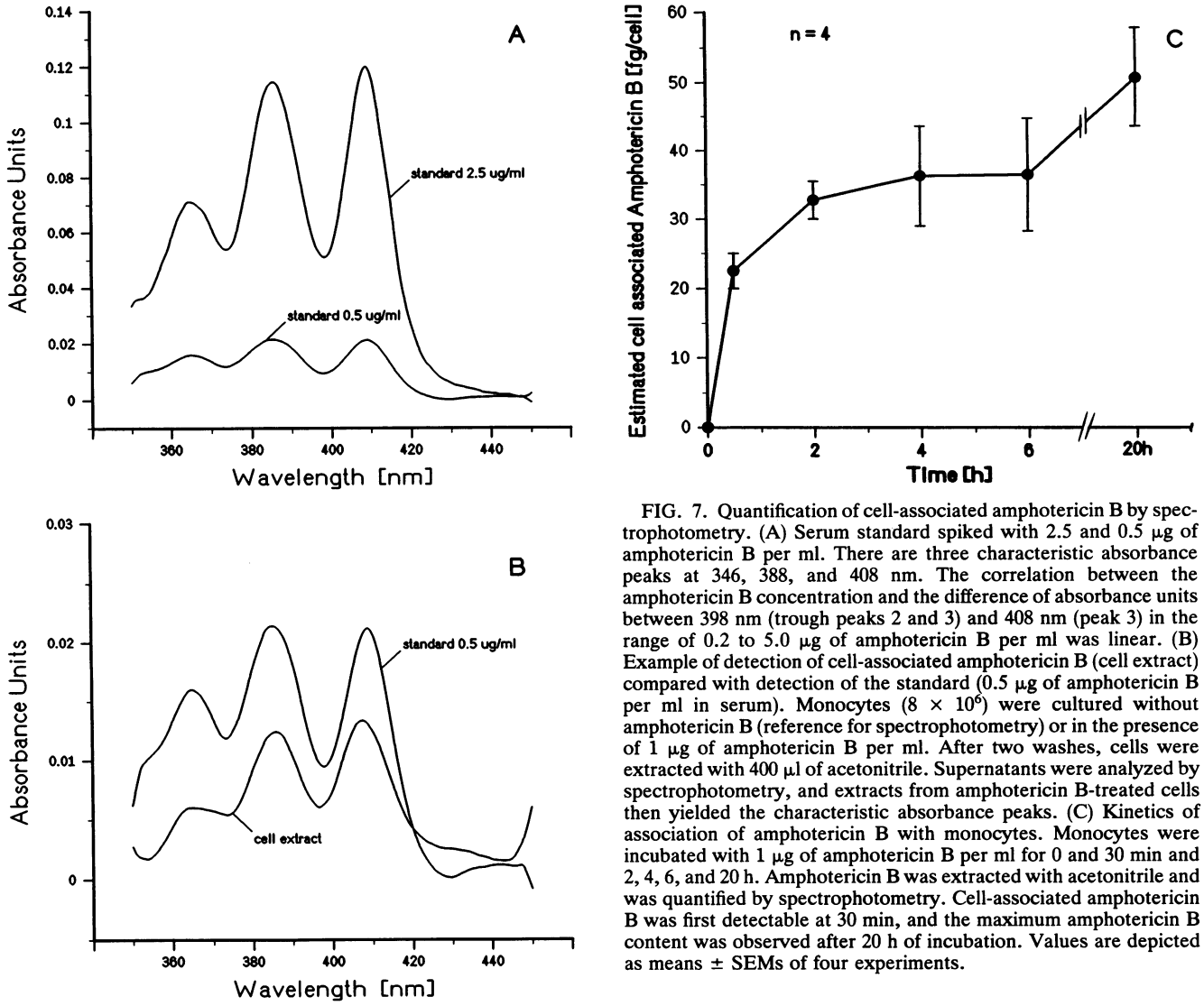

FIG. 7. Quantification of cell-associated amphotericin B by spectrophotometry. (A) Serum standard spiked with 2.5 and 0.5 μg of amphotericin B per ml. There are three characteristic absorbance peaks at 346, 388, and 408 nm. The correlation between the amphotericin B concentration and the difference of absorbance units between 398 nm (trough peaks 2 and 3) and 408 nm (peak 3) in the range of 0.2 to 5.0 μg of amphotericin B per ml was linear. (B) Example of detection of cell-associated amphotericin B (cell extract) compared with detection of the standard (0.5 μg of amphotericin B per ml in serum). Monocytes (8×10^6) were cultured without amphotericin B (reference for spectrophotometry) or in the presence of 1 μg of amphotericin B per ml. After two washes, cells were extracted with 400 μl of acetonitrile. Supernatants were analyzed by spectrophotometry, and extracts from amphotericin B-treated cells then yielded the characteristic absorbance peaks. (C) Kinetics of association of amphotericin B with monocytes. Monocytes were incubated with 1 μg of amphotericin B per ml for 0 and 30 min and 2, 4, 6, and 20 h. Amphotericin B was extracted with acetonitrile and was quantified by spectrophotometry. Cell-associated amphotericin B was first detectable at 30 min, and the maximum amphotericin B content was observed after 20 h of incubation. Values are depicted as means ± SEMs of four experiments.

tissues of patients undergoing amphotericin B therapy. It is probable that intracellular accumulation is a general property not restricted to monocytes and macrophages. However, accumulation in the latter is of consequence because these cells take up the fungi and the drug is available to kill the phagocytosed yeasts.

Alveolar macrophages play an important role in preventing fungal infections, and the levels of amphotericin B in pulmonary tissue that can be expected to produce an augmented killing capacity should easily be achieved by local administration of the drug, e.g., via aerosol, which may circumvent systemic side effects. Systemic administration of low doses of amphotericin B also appears to exhibit prophylactic efficacy with regard to pulmonary infections (29). It will be of interest to determine whether the monocytes and macrophages of such patients have higher fungicidal capacities ex vivo.

To our knowledge, the remarkable potentiation of the candidacidal activity of human monocytes by amphotericin B has not been noted previously. Perfect et al. (26) did report in 1987 that mouse macrophages preincubated with amphotericin B for 4 h exhibited an augmented killing capacity toward a tumor cell line and also an enhanced killing of *Candida parapsilosis* or *Cryptococcus neoformans*. Those experiments differed from ours in two respects. First, the period of incubation with amphotericin B was only 4 h. In our experience, this was the period at which clear augmented killing of *C. albicans* began to be noted. Second, the concentrations of amphotericin B that they used were much higher. Thus, data showing augmented killing of fungi were obtained with 5 μg of the agent per ml. Because of the tumoricidal effects, the conclusion was that amphotericin B acted via stimulation of the macrophages.

Pascual et al. (25) observed enhanced killing of yeasts by macrophages in the presence of amphotericin B. In that report, the drug was added after uptake of yeasts. Augmentation of yeast killing required very high amphotericin B concentrations (10 μg/ml) and may have been due to a direct fungicidal effect of the drug. A few publications contain data that indirectly point to an intracellular accumulation of the drug. Newman et al. (21) applied amphotericin B to human monocytes after the cells had phagocytosed *Histoplasma capsulatum*. They reported that digestion of the fungi during 24 h was enhanced in the presence of low doses of amphotericin B. Van Etten et al. (33) reported similar experiments

with *C. albicans* and murine macrophages and found extensive killing of phagocytosed fungi during 6 h of incubation with 0.4 μg of amphotericin B per ml. Their experimental approach and results most closely resembled our present findings; accumulation of the drug in the macrophages was not demonstrated. Bistoni et al. (3) reported enhanced killing of *C. albicans* after preincubation of murine macrophages with 2.5 or 10 μg of amphotericin B per ml. Lower doses were not used in their study. Those authors also interpreted their data to indicate activation of the macrophages by amphotericin B.

We wish to mention that experiments were also performed with human neutrophils as effector cells. The cells were preincubated for 6 h with 0.1 to 0.2 μg of amphotericin B per ml prior to challenge with *C. albicans*. Alternatively, phagocytosis and killing assays were performed in the presence of 0.2 μg of amphotericin B per ml for 6 h. However, no enhancement of killing was discernible in those experiments. In this context, it is noteworthy that the intracellular accumulation of fluconazole (25) and itraconazole (27) has been reported. Whereas accumulation of itraconazole appeared to enhance the fungicidal capacity of macrophages, a comparable effect was not noted with fluconazole and neutrophils. These findings show some analogy to our results with amphotericin B. The apparent failure of both amphotericin B and fluconazole to enhance the killing of *C. albicans* by neutrophils may be related to the fragility and short life span of these effector cells. At present, it thus remains unknown whether the observations made in the present study might extend to human neutrophils in an in vivo situation.

Conclusion. Monocytes and macrophages incubated with low, nontoxic doses of amphotericin B accumulate the drug. This endows the cells with a markedly enhanced capacity to kill amphotericin B-susceptible *C. albicans*. Presumably, similar enhancement of killing will also occur with other yeasts that are susceptible to the drug. The described phenomenon may be partly responsible for the curative effect and prophylactic efficacy of this most widely used antifungal agent.

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