Amphotericin-B Promotes Leukocyte Aggregation of Nylon-Wool-Fiber-Treated Polymorphonuclear Leukocytes

By Laurence A. Boxer, Leah M. Ingraham, John Allen, Ronald S. Oseas, and Robert L. Baehner

Severe pulmonary reactions have been reported in patients receiving leukocyte transfusion and amphotericin-B. To study the interaction of amphotericin-B with polymorphonuclear leukocytes (PMN), purified human PMN were incubated with 200 mg of nylon wool fiber for 60 min either in the absence or presence of 2 mM EDTA. PMN were recovered in acid citrate dextrose solution and were suspended in balanced salt solution for determination of their aggregation properties. The cells exposed to nylon wool fibers without EDTA aggregated in response to concentrations as low as 1.25 µg/ml of amphotericin-B. Cells initially treated with EDTA, however, failed to aggregate. Serum from a patient treated with amphotericin-B aggregated PMN exposed to nylon wool fiber but not control cells, whereas serum taken before amphotericin-B administration was without effect on the PMN treated with nylon wool fiber. Amphotericin-B at 5 µg/ml failed to potentiate the release of beta-glucuronidase or lactic dehydrogenase by PMN treated by nylon wool beyond that seen with exposure to the fibers alone. Rabbit peripheral blood was similarly incubated with nylon wool fibers and the recovered PMN were infused into recipient rabbits that had received 1 mg/kg of amphotericin-B intravenously 1 hr prior to the infusion of the leukocytes. Rabbits were sacrificed 30 min after transfusion of PMN, and their lungs were excised for histologic sectioning. Those rabbits receiving a combination of amphotericin-B and 4 × 10^7 nylon-wool-fiber-treated PMN had evidence of pulmonary hemorrhage and accumulation of leukocytes in the pulmonary vasculature whereas those animals who received such cells alone had normal appearing lung tissue. In summary, amphotericin-B at concentrations achievable in vivo enhanced the aggregation of PMN damaged by incubation with nylon fiber with subsequent accumulation of the phagocytes in pulmonary tissue.

LETHAL PULMONARY REACTIONS associated with the simultaneous use of amphotericin-B and leukocyte transfusions in high-risk patients with profound granulocytopenia and suspected sepsis have recently been described in one study but not another. Over 50% of the patients receiving the combined therapeutic modalities in the former study had respiratory deterioration with pulmonary infiltrates seen on chest x-ray and pulmonary damage. On histologic examination of lung tissue obtained by biopsy or autopsy, intraalveolar hemorrhage was found. These clinical observations are reminiscent of the findings in patients with respiratory distress syndrome of adults whose serum initiates leukoaggregation in vitro and in whom pulmonary hemorrhage is observed in vivo. We therefore considered the possibility that amphotericin-B could promote aggregation of nylon-wool-fiber-treated (NWF) polymorphonuclear leukocytes (PMN) in vitro and possibly initiate toxic pulmonary reactions in rabbits following administration of amphotericin-B and leukocyte transfusions. In this study we found that amphotericin-B potentiated aggregation of PMN exposed to NWF and that plasma obtained from a patient receiving amphotericin-B similarly affected PMN aggregation. In addition, the combination of amphotericin-B and leukocytes harvested from NWF elicited pulmonary damage in rabbits.

MATERIALS AND METHODS

Isolation

Purified human PMN of greater than 98% purity with less than 1 platelet/1000 cells were isolated from heparinized venous blood drawn from normal human volunteers taken in accordance with the principles of the Declaration of Helsinki. Prior to dextran sedimentation, 1 mM adenosine diphosphate (ADP) was added to the heparinized blood and agitated for 2 min. Next, dextran (6%) in 0.9% NaCl was added to the whole blood, and the syringe was placed upright. The red cells and platelets (which were aggregated with the addition of the ADP) were allowed to settle out and the leukocyte-rich supernate was removed from the top of the syringe. The leukocyte-rich plasma was then layered on a Ficoll-Hypaque gradient and centrifuged at 400 g for 30 min at 4°C. The supernate was discarded, and the cell pellet subjected to hypotonic lysis for 20 sec to remove contaminating erythrocytes. The purified human PMN were suspended in Kreb's Ringer phosphate buffer pH 7.4 (KRP) at a concentration of 10^7 cells/ml. Peritoneal PMN were obtained from New Zealand white rabbits 18 hr after an intraperitoneal injection of 100 ml of 12% sodium caseinate. Greater than 90% of the cells obtained in this manner were PMN as determined by Wright stain smear. Cells were centrifuged at 250 g for 10 min at 4°C and washed twice with KRP, pH 7.4. The PMN were resuspended following the second centrifugation in KRP, pH 7.4, at a concentration of 10^7 cells/ml. NWF was packed into a 20-ml plastic syringe. Ten milliliters of the PMN suspension was allowed to
incubate with 200 mg of NWF for 1 hr. The PMN were removed by passing 600 ml of an acid citrate dextran-plasma solution (ACD-P) over the nylon wool fibers, while the syringe barrel was gently tapped. All of these procedures were carried out at room temperature. The eluted cells were then resuspended in phosphate-buffered saline, pH 7.4.

In order to simulate the isolation of PMN collected by continuous-flow centrifugation,1 leukocytes consisting of 96% purity were isolated from heparinized whole blood by counterflow centrifugation elutriation88 and suspended in KRP at a concentration 10’ cells/ml.

Aggregometry

Aggregometry was performed as a modification of methods described by Caddock et al.44,45. A standard platelet aggregometer recording system (Chronolog, Model 300) was used. To a siliconized cuvette containing a Teflon stirrer revolving at 900 rpm, 0.45 ml of a suspension containing 1.5 x 10’ PMN/ml was added. Both magnesium and calcium were added in a final concentration of 1.2 mM. After a 2-min delay to allow warming of cells to 37°C, 50 µl containing n-formyl-l-methionyl-l-leucyl-l-phenylalanine (FMLP) (Peninsula Lab, Palo Alto, Calif.) or amphotericin suspended in 0.9% NaCl (Grand Island Biological Co., Inc., Grand Island, N.Y.) was added and the resulting changes in light transmission recorded as ΔT. The amphotericin was in powdered form and free of the dispersing agent sodium deoxycholate. To provide the necessary amplification for a well defined aggregation wave, the aggregometer recording system was calibrated with fresh PMN suspension diluted to a 40% volume with KRP. Quantitation of PMN aggregation during the initial 3 min was made using a Lietz compensating optical polar planimeter (Model 3651–30) and results expressed in sq cm. The variability and total aggregation by PMN during the 3-min interval did not exceed 15% on the same day or on subsequent days. The Student’s t test was used employing the areas obtained from triplicate aggregation.

In Vivo Studies

Ten milliliters of heparinized whole blood was obtained from rabbits and allowed to incubate with 200 mg of nylon wool fiber for 1 hr at room temperature. The adherent cells were eluted from the NWF by passing 60 ml of ACD-P over the fiber and by gently tapping the syringe. The eluted cells were centrifuged at 250 g and then resuspended in KRP at a concentration of 10’ PMN/ml, and 4 x 10’ PMN were infused into recipient rabbits that had previously received 1 mg/kg of amphotericin-B or 1 ml/kg of rabbit serum intravenously 1 hr prior to the leukocyte infusion. Thirty minutes following the infusion of the leukocyte suspension, the rabbits were killed with sodium nembutal and the lungs were immediately removed and placed in 0.9% NaCl (Grand Island Biological Co., Inc., Grand Island, N.Y.) and which were placed in a syringe holding NWF or media devoid of EDTA. AMB was also used as a stimulus for normal PMN aggregation on in vivo was employed to ascertain whether the drug would aggregate nylon-wool-fiber-exposed PMN suspended in KRP. As seen in Table 1, amphotericin-B aggregated PMN in balanced salt solution in a dose-dependent fashion. On the other hand, amphotericin-B did not aggregate PMN that had been treated with the divalent cation chelator EDTA, nor did phosphate-buffered saline aggregate PMN exposed to nylon wool fiber; these facts imply that divalent cations are necessary for aggregation to occur. In addition, amphotericin-B aggregates damaged PMN.

Fluorescence Measurements

All fluorescent measurements were made in a Perkin-Elmer MPF-44B fluorescent spectrophotometer (Perkin-Elmer Corp. Instrument Div., Norwalk, Conn.) equipped with a temperature-controlled cuvette holder and automatic stirrer. Chlortetracycline (CTC), 10 µM, was added to the cell suspension (3 x 10’ PMN/ml in HEPES buffer, 10 mM, pH 7.35, with 130 mM NaCl, 5 mM KCl, and 100 mg/100 ml glucose). In experiments with exogenous calcium present, CaCl2 (1.0 mM) was added just before the CTC. The cell suspensions were incubated for 30 min at 37°C, during which time the CTC associated with the cell membrane.11 Two milliliters CTC-labeled cell suspension were placed in a cuvette and the baseline fluorescence determined. Excitation was at 390 nm and emission at 530 nm. Slit widths were between 6 and 8 nm. The various addition (amphotericin, 1 µg/ml and/or FMLP, 5 x 10’ M) were made at the desired time, and the fluorescence was monitored continuously via a recorder.

RESULTS

To study the influence of amphotericin-B on PMN aggregation, amphotericin-B at doses achievable in vivo was employed to ascertain whether the drug would aggregate nylon-wool-fiber-exposed PMN suspended in KRP. As seen in Table 1, amphotericin-B aggregated PMN in balanced salt solution in a dose-dependent fashion. On the other hand, amphotericin-B did not aggregate PMN that had been treated with the divalent cation chelator EDTA, nor did phosphate-buffered saline aggregate PMN exposed to nylon wool fiber; these facts imply that divalent cations are necessary for aggregation to occur. In addition, amphotericin-B

<table>
<thead>
<tr>
<th>Addition</th>
<th>Aggregation Response (sq cm)</th>
<th>p Value</th>
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<tbody>
<tr>
<td>Phosphate-buffered saline to NWF-treated cells</td>
<td>5.4 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>5 µg/ml AMB to EDTA-treated cells</td>
<td>5.2 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>0.63 µg/ml AMB to NWF-treated cells</td>
<td>6.8 ± 0.3</td>
<td>&lt;0.025</td>
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<td>1.25 µg/ml AMB to NWF-treated cells</td>
<td>8.6 ± 0.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>5.0 µg/ml AMB to NWF-treated cells</td>
<td>11.4 ± 0.5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>2 x 10^-7 M FMLP to control PMN treated with CB</td>
<td>24.9 ± 0.89</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2 x 10^-7 M FMLP to NWF-treated cells</td>
<td>20.8 ± 0.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>5 µg/ml AMB to control PMN</td>
<td>5.7 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>2 µg/ml AMB to aged elutriated cells treated with CB</td>
<td>14.0 ± 0.1</td>
<td>&lt;0.01</td>
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Quantitation of human PMN aggregation over 3 min was performed following the addition of 50 µl of the aggregate containing amphotericin-B (AMB) to cells originally suspended in media containing 2 mM EDTA and which were placed in a syringe holding NWF or media devoid of EDTA. AMB was also used as a stimulus for normal PMN aggregation on elutriated PMN, which sat at room temperature for 3 hr and then were treated with 5 µg/ml cytochalasin-B (CB) at 37°C for 5 min. FMLP was employed as a stimulus for NWF-treated PMN or normal PMN pretreated for 5 min at 37°C with 5 µg/ml cytochalasin-B. A polarimeter was used to measure the area under the aggregation curve. Student’s t tests were done employing the areas obtained from triplicate aggregation curves.

Table 1. Effect of Amphotericin-B on Nylon-Wool-Fiber-Treated PMN
cin-B failed to effect aggregation of PMN not exposed to nylon wool fiber; whereas these same cells were responsive to the known aggregant, FMLP, at a concentration of $2 \times 10^{-7}M$. Under the latter conditions, the NWF-treated PMN responded to FMLP like normal PMN treated with cytochalasin-B. Although not shown, 5 μg/ml amphotericin-B failed to aggregate fresh PMN obtained by elutriation and pretreated with cytochalasin-B but could aggregate elutriated PMN that sat at room temperature for 3 hr and had been exposed to cytochalasin-B (5 μg/ml) for 5 min (Table 1).

Since amphotericin-B aggregated NWF-treated PMN directly, we wished to determine if serum containing amphotericin-B would aggregate NWF-treated PMN. Thus, serum was obtained from a patient 90 min after the infusion of 1.0 mg/kg of amphotericin-B. As observed in Fig. 1, the postinfusion serum aggregated the NWF-treated PMN; whereas the preinfusion serum, like control serum, failed to initiate aggregation of NWF-treated PMN.

Since PMN that have degranulated readily aggregate, we considered the possibility that amphotericin-B might potentiate PMN degranulation beyond that observed for PMN exposed to NWF alone. As indicated in Table 2, exposure to NWF provoked release of both β-glucuronidase and LDH. Amphotericin-B failed to potentiate the release of β-glucuronidase beyond that observed with exposure of the PMN to NWF alone. Furthermore, amphotericin-B was not toxic to the PMN, because LDH release was no greater than that observed for PMN exposed to NWF alone.

Based on the in vitro findings we considered the possibility that the combination of amphotericin-B administered along with NWF-treated PMN might be toxic in vivo because there is reasonable correlation between the formation of leukoaggregates in vitro following chemotactic stimuli and subsequent pulmonary damage in vivo. In order to evaluate whether amphotericin-B might lead to pulmonary damage, three rabbits were infused with $4 \times 10^7$ PMN of NWF-treated PMN after receiving 1 mg/kg of amphotericin-B 1 hr prior to the leukocyte transfusion. Thirty minutes later, the animals were sacrificed and the lungs were excised and examined histologically. As noted in Fig. 2, the lungs of the animals receiving NWF-treated PMN failed to show any damage or accumulation of PMN in the vessels. In contrast (Fig. 3), the animals receiving both amphotericin-B and NWF-treated PMN invariably developed pulmonary edema and hemorrhage and formed intravascular leukoaggregates. Treatment of the rabbits with amphotericin-B followed by infusion of the NWF-treated PMNs likely failed to activate the complement system because the total hemolytic complement in the animals' sera remained unchanged following the infusion of amphotericin and NWF-treated cells.

One membrane-mediated response that occurs upon FMLP stimulation of PMN is the translocation of membrane-bound calcium. This effect can be monitored by use of fluorescent dye (CTC), which asso-
AMPHOTERICIN-B AGGREGATES DAMAGED PMN

In an effort to gain a greater understanding of the differences between untreated and NWF-treated cells, we examined them for the ability to take up CTC and to respond to FMLP in the presence of amphotericin. NWF-treated cells showed only 50%-70% the fluorescence of control cells when labeled with CTC (10 μM) (Table 3). Since diminished fluorescence is found whether calcium is absent or present in the external medium, it appeared that there may be membrane alterations that prevented CTC membrane association in NWF cells rather than decreased calcium concentration in the membrane of such cells.

Both control and NWF cells labeled with CTC showed a loss of fluorescence when amphotericin (1 μg/ml) was added (Table 3), although NWF-treated cells had less of a loss than did the control PMN. When FMLP (5 x 10^-7 M) was added after the addition of amphotericin, however, both control and

NWF-treated cells exhibited a characteristic loss of fluorescence (Table 3). Therefore, despite membrane alterations, the NWF cells remained capable of translocating calcium upon chemotactic stimulation, and amphotericin at 1 μg/ml neither inhibited nor promoted that response.

DISCUSSION

Amphotericin-B has remained the drug of choice for the treatment of systemic fungal infections because of its relatively high affinity for combination with the sterols of the plasma membrane of the fungal cells compared to those of animal cells membranes, thereby leading to “leakage” of cytoplasmic contents from the fungi and their ultimate demise. The usual dosage is 0.5–1.0 mg/kg daily or up to 1.5 mg every other day, the total dose depending on organism response and patient tolerance. The drug is slowly excreted, which contributes to blood levels of 2–3 μg/ml achievable with maximal dose of the drug. Its toxic effects in patients have been extensively reviewed and can be summarized as follows: (1) renal toxicity manifested as azotemia, (2) hematopoietic toxicity manifested as anemia and thrombocytopenia, (3) electrolyte abnormalities chiefly hypokalemia, (4) rare instances of hepato- and cardiotoxicity, and (5) immediate side effects of chills, fever, anorexia, headache, nausea, vomiting, and phlebitis. In addition to these known side effects, it now appears that amphotericin-B may damage PMN, leading to their subsequent aggregation and possible toxic pulmonary reactions.

These latter observations are reminiscent of the pathophysiology that may underlie the adult respiratory distress syndrome. It is known that activated complement fragments as well as synthetic oligochemotactic peptides can induce PMN to autogaggregate in vitro. Moreover, the same stimuli may engender PMN to obstruct small blood vessels followed by tissue injury in vivo. In these studies we observed that both amphotericin-B in concentrations which can be achieved in vivo and the serum of patients receiving amphotericin-B could incite NWF-treated PMN to aggregate. Damage to the PMN by nylon fibers was a prerequisite for aggregation to occur, because amphotericin-B failed to aggregate either normal or freshly obtained elutriated PMN or PMN previously suspended in EDTA. Like others, we found that PMN eluted from NWF lost substantial quantities of their cellular contents as evidenced by leakage of the cytoplasmic enzyme LDH and the granule enzyme beta-gluconoridase. Addition of amphotericin-B for 5 min to the nylon-fiber-treated-PMN failed to potentiate enzyme release.

The question remains as to how amphotericin-B...
promotes aggregation of NWF-treated PMN. Various cell types differ in their susceptibility to amphotericin B, and the outcome of exposure to the drugs is most influenced by the concentration of the drug and by the time and temperature of incubation. Alterations of cell function may follow binding of amphotericin-B. Both B and T lymphocytes exhibit altered properties in presence of the drug. Differential effects of the polyclonal antibodies on different cell types has also been documented. For example, transformed 3T3 cells treated with filipin show greater tendency to lysis, altered response to wheat germ agglutinin, and marked changes in morphology; untransformed cells, however, fail to show these pronounced effects. Furthermore, amphotericin effects on 3T3 cells differ depending on whether the cells were in interphase or were undergoing mitosis. The above results along with ours suggest that the state of the cell membrane at time of amphotericin-B incubation may be crucial in determining the subsequent effects of the drug. We found that amphotericin-B would promote PMN aggregation if the cells had been exposed to NWF or if the cells had been prepared by elutriation followed by exposure to room temperature and cytochalasin-B. Our work with chlorotetracyline (CTC) suggests that the NWF-treated cells have membrane properties different from those of untreated PMN, and most likely membrane changes occur in elutriated cells when they are treated with cytochalasin-B.

We found that less CTC was taken up by NWF-treated cells. Their fluorescence was less than that of control cells when incubated in 10 μM CTC, and this effect appeared not to arise from lack of calcium because addition of calcium to the medium did not bring NWF-treated PMN fluorescence to normal levels. Accordingly, we suggest that the membrane sites usually occupied by CTC may be altered by NWF exposure.

Such membrane alterations could also figure in the hyperaggregability of NWF-treated cells exposed to amphotericin-B. It does not appear that amphotericin-B alters the cells' ability to respond to aggregating stimuli. We showed that the calcium translocation, which characteristically follows FMLP stimulation, was essentially normal in NWF cells when exposed to levels of tripeptide that promote aggregation. It seems more likely then that some change has occurred in membrane structure that allows amphotericin-B to potentiate cell–cell interaction. Similarly, under conditions of aging, when the PMN is leaking calcium leading to alterations in cell surface charge, which can be exacerbated by the addition of cytochalasin-B, amphotericin induced aggregation of the cells.

Agents that reduce cell surface charge promote leukoaggregation. Increased hydrophobicity of a cell membrane should also enhance cell association. Perhaps amphotericin-B brings about changes in cell surface charge and/or hydrophobicity through its association with membrane constituents. The drug binds specifically and stoichiometrically to membrane cholesterol, rearranging the cholesterol into complexes. Such rearrangements might have profound effects on the biochemical and biophysical properties of the membrane surface. However, until more is known about the mechanisms by which PMN adhere to one another, it will be impossible to say how amphotericin-B promotes aggregation.

The initial observations noted that the toxic pulmonary reactions in patients receiving amphotericin-B occurred during the combined administration of the drug along with PMN obtained by centrifugation. When freshly obtained, PMN will not readily degranulate and remain in suspension. However, prolonged incubation of these cells even at room temperature may lead to calcium loss, thereby making the PMN more vulnerable to amphotericin-B-mediated alterations in motile function and susceptible to agglutination. Based on these observations, it would be our recommendation to administer amphotericin-B and freshly obtained centrifuged white cells separately, at widely spaced intervals, to decrease the likelihood of these toxic reactions.

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