Pharmacologic Modulation of Interleukin-1 Expression by Amphotericin B-Stimulated Human Mononuclear Cells

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Fever and chills occur frequently with amphotericin B (AB) administration, but the mechanism that causes these reactions has not been definitively established. A variety of proinflammatory cytokines, including interleukin-1 (IL-1) and tumor necrosis factor, have been shown to be important mediators of fever. In order to clarify the cellular and biochemical responses associated with AB-induced fever, the experiments described sought to (i) establish whether human mononuclear cells exposed to AB in vitro expressed IL-1β, (ii) evaluate whether clinically used premedications for fever prophylaxis in AB-treated patients were effective in down-regulating IL-1β expression in vitro, (iii) evaluate whether methylnitine agents with immunomodulatory actions affected in vitro IL-1β expression, and (iv) define the dose and time dependency of the modulating effects. Peripheral blood mononuclear cells were isolated by density centrifugation and resuspended to 10^6 cells per ml in culture wells of Linbro plates. When cocultured for 2 h with human mononuclear cells, both Escherichia coli lipopolysaccharide and AB stimulated IL-1β expression in a dose-related fashion. AB-induced IL-1β expression was suppressed by hydrocortisone (HC), pentoxifylline, and an investigational theobromine, A81-3138, in a linear, dose-related manner. In contrast, indomethacin, meperidine, and diphenhydramine had no effect on IL-1β expression. Our in vitro data indicate that serum HC concentrations of greater than 1 to 2 μg/ml may be sufficient to modulate IL-1β expression. Pentoxifylline and A81-3138 may also be effective in modulating IL-1β expression by mononuclear cells at concentrations achievable in serum. These new agents may prove to be effective alternatives to HC or may be added with HC to suppress febrile reactions secondary to AB administration. Clinical studies with pentoxifylline as a premedication for AB seem warranted.

Amphotericin B (AB) is an intravenous agent used in the treatment of severe fungal infections. Its usefulness is compromised by a high incidence of adverse effects. Fever and chills are observed in up to seventy percent of treated patients (3). The mechanism responsible for this reaction is unknown. The proposed mechanisms of AB-induced febrile reactions include the expression of interleukin-1 (IL-1), tumor necrosis factor (TNF), or prostaglandins by mononuclear cells (MNCs), which then alter the hypothalamic set point, inducing fever and chills (2, 8, 9). Administration of endotoxin causes similar reactions. The goals of the studies described here were (i) to establish whether human mononuclear cells exposed to AB in vitro expressed IL-1β, (ii) to evaluate whether premedications (hydrocortisone, indomethacin, diphenhydramine, meperidine) used clinically for fever prophylaxis in AB-treated patients are effective in down-regulating IL-1β expression in vitro, (iii) to evaluate whether methylnitine agents with immunomodulatory effects would affect in vitro IL-1β expression, and (iv) to define the dose and time dependency of these modulating effects.

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MATERIALS AND METHODS

Reagents. Unsupplemented medium (RPMI 1640) was obtained from Flow Laboratories (McLean, Va.). The pharmacologic agents used in this study included commercial-grade AB (Fungizone; Squibb, Princeton, N.J.), Escherichia coli endotoxin (serotype O26:B6 lipopolysaccharide [LPS]; Sigma, St. Louis, Mo.), hydrocortisone (HC; Elkins-Sinn, Cherry Hill, N.J.), indomethacin (IDM; Merck Sharp & Dohme, Rahway, N.J.), diphenhydramine (DPH; Elkins-Sinn), meperidine (MEP; Wyeth-Ayerst Laboratories, Philadelphia, Pa.), pentoxifylline (PTF; Hoechst-Roussel Pharmaceuticals, Somerville, N.J.), and A81-3138 (A81; Hoechst-Roussel Pharmaceuticals). Deoxycholate and sodium phosphate buffer were obtained from Sigma. Reagents were diluted with sterile water so that 0.01 ml of stock solutions added to culture wells resulted in the final concentrations noted throughout this report.

Samples of diluent (sterile water), media, reagents, and flushed wells from culture plates were assayed for endotoxin by the Limulus amebocyte lysate assay (Associates of Cape Cod Inc., Woods Hole, Mass.). This assay uses an E. coli endotoxin standard and had a lower limit of detection of 20 pg/ml during the initial phases and 3.0 pg/ml during the later phases of the studies described in this report. None of the reagents that were screened were positive for endotoxin. Media harvested from cell-free wells of culture plates were also negative for endotoxin.

MNC preparation. Heparinized blood was collected from normal healthy human volunteers by peripheral venipuncture. MNCs were sedimented in Mono-Poly resolving medium (Ficoll-Hypaque; Flow Laboratories) removed by use of a pipet, and washed three times in unsupplemented medium. The average cell yield from 90 ml of blood was 1.0 x 10^9 to 2.0 x 10^9 cells with greater than 95% viability.
(established by Trypan blue exclusion). A nonspecific esterase revealed an average of 66.3% ± 16.2% lymphocytes and 31.0% ± 11.7% monocytes. MNCs were resuspended to a final concentration of approximately 5 × 10⁶ cells per ml in supplemented medium (RPMI 1640, 10% autologous serum, 100 µg of streptomycin per ml, and 100 U of penicillin per ml). One milliliter of MNCs was seeded in Linbro 24-well plates (Flow Laboratories) and incubated for 1 h at 37°C in 5% CO₂ before the experimental study.

**Experimental design.** (i) **AB- and LPS-stimulated cultures.** After MNC preparation, cells were exposed to AB or LPS at concentrations ranging from 0.0325 to 10.0 µg/ml and 0.0001 to 0.1 µg/ml, respectively. In time studies, MNCs were incubated with a fixed concentration of AB or LPS from 0.5 to 48 h before the samples were harvested. For dose-response and pharmacologic modulation studies, a 2-h incubation was chosen because significant increases in IL-1β were noted in all experiments of 2 h or longer, and this period represented the window of time (60 to 180 min) when AB-induced fevers were observed in vivo.

To evaluate the potential additive, synergistic, or antagonistic effects of LPS and AB on MNC IL-1β expression, in a limited number of experiments, submaximal concentrations of LPS (0.0001 to 0.001 µg/ml) and AB (0.625 to 2.5 µg/ml) were used as simultaneous stimulants.

(ii) **Pharmacologic modulation of IL-1β expression.** After cell preparation, MNC cultures were exposed to LPS (0.0001 or 0.001 µg/ml) or AB (1 or 2 µg/ml). In duplicate wells, HC (0.001 to 2.0 µg/ml), IDM (0.01 to 10 µg/ml), DPH (0.001 to 1.0 µg/ml), MEF (1.0 to 10 µg/ml), PTF (0.1 to 1000 µg/ml), or A81 (6.25 to 1,000 µg/ml) was added simultaneously with the stimulants to the culture. MNCs were then incubated for 2 h before the samples were harvested.

In other experiments, MNC cultures were preincubated with HC (0.5 µg/ml) or PTF (100 µg/ml) for 1 h and 45, 30, and 15 min before the addition of AB (1 or 2 µg/ml) or LPS (0.0001 or 0.050 µg/ml). MNCs were then incubated for 2 h before the samples were harvested.

(iii) **Harvesting of IL-1β-rich supernatants.** After completion of each experiment, MNCs were exposed to three freeze-thaw cycles in order to lyse the cell membranes (7). Three hundred microliters from each well was collected and stored at −70°C until it was assayed for IL-1β. All conditions were tested in duplicate.

(iv) **Control experiments.** MNCs were evaluated for their viabilities and morphologies at the completion of randomly selected studies. Positive control wells contained MNCs and AB (1 to 2 µg/ml) or LPS (0.0005 µg/ml). Negative control wells included cells with medium alone, deoxycholate, HC, IDM, DPH, MEF, PTF, A81, or sterile water.

(v) **IL-1β assay.** Samples were assayed for IL-1β by using an enzyme-linked immunosorbent assay (ELISA; Cistron Biotechnology, Pine Brook, N.J.). The procedure involves a four-step test carried out in microtiter wells which are coated with IL-1β-specific monoclonal antibody. Manufacturer’s data indicate an assay sensitivity of 20.0 pg/ml and a specificity for IL-1β. There was no cross-reactivity for IL-1α, IL-2, TNF, or interferon. Evaluations of assay precision demonstrated a coefficient of variation of 5.3 to 6.7% for intraassay variability and 6.6 to 8.4% for interassay variability. Data are the means of duplicate assays and are expressed in picograms per milliliter on the basis of standards supplied by the manufacturer. During the initial phases of our study, a bioassay with the LBRM 33-IAS cell line was performed as described by Gillis and Mize (10). Similar results were obtained by both the bioassay and ELISA.

**Statistical analysis.** Curves were fit to datum points by using computer software which generates a line of best fit by a quantal dose-effect model (Slide Tek, Saulsalito, Calif.). Data were evaluated by analysis of variance. Once significance was determined, values were then compared by using a post hoc multiple range test (Student-Newman-Keuls test). The Student-Newman-Keuls test allowed for evaluation of the multiple variables to determine which values were different from the values for the controls.

**RESULTS**

**AB- and endotoxin-stimulated IL-1β expression.** In order to clarify the kinetics of IL-1β expression, AB (at a fixed concentration of 2.0 µg/ml) was incubated with MNCs for 48 h. The greatest incremental IL-1β increases were observed during the first 10 h of incubation and reached a plateau thereafter, as follows (n = 5; concentration ± standard error of the mean): 0.5 h, 60.9 ± 1.27 pg/ml; 1.0 h, 61.5 ± 0.89 pg/ml; 2 h, 279.4 ± 54.0 pg/ml; 4 h, 1,013.0 ± 51.2 pg/ml; 10 h, 1,110.0 ± 111.9 pg/ml; 20 h, 1184.9 ± 223 pg/ml; 24 h, 1,179.0 ± 99 pg/ml; 36 h, 1,134.2 ± 165.4 pg/ml; 48 h, 1,406.6 ± 181.2 pg/ml. Simultaneous normal saline controls were always less than 117.5 ± 67.1 throughout the 48 h. In all experiments, AB-stimulated IL-1β concentrations after 2 h or longer of incubation were significantly greater than simultaneous concentrations in controls. When they were incubated with MNCs for 2 h, both LPS and AB stimulated MNC IL-1β expression in a dose-dependent manner for MNCs from all five volunteers studied (Fig. 1). Either AB (1 to 2 µg/ml) or LPS (0.0001 to 0.0005 µg/ml) resulted in the expression from MNCs of 300 to 600 pg of IL-1β per ml. Use of medium with deoxycholate, HC, MEF, IDM, DPH, PTF, A81, or diluent alone did not result in a significant increase in IL-1β expression over that in controls (P > 0.1). Inspection of monolayers by light microscopy revealed no effect on cell morphology or viability at all concentrations of LPS and at AB concentrations of less than 5 µg/ml. Cell viability was reduced by 10 to 15% with AB concentrations of 10 µg/ml.

In limited experiments that were done to evaluate the potential additive, synergistic, or antagonistic effects of LPS and AB on MNC IL-1β production, suboptimal concentrations of LPS and AB were used as simultaneous stimulants.
No reproducible or significant additive, synergistic, or antagonistic effect could be established in these experiments (data not shown).

**Pharmacologic modulation of IL-1β expression.** AB at 1 or 2 μg/ml was selected for use in the experimental studies described here, since these concentrations of AB are achievable in the sera of humans, significantly stimulated IL-1β production in all experiments, produced responses similar to those of LPS, and did not affect MNC viability. Hydrocortisone, PTF, and A81 produced dose-related suppression of IL-1β expression by AB-stimulated MNCs in all five experimental protocols evaluated (Fig. 2). When similar experiments were performed with LPS as a stimulus, HC and PTF (A81 was not tested) also suppressed IL-1β expression in a dose-related fashion (data not shown). At a peak concentration of 1 μg of HC per ml, AB-induced IL-1β expression was suppressed to 39.0% ± 6.4% (standard error of the mean [SEM]) of the maximum response. At peak concentrations of PTF (1,000 μg/ml) and A81 (1,000 μg/ml), AB-induced IL-1β expression was suppressed to 21.3% ± 8.3% (SEM) and 18% ± 9.1% (SEM) of the maximum response. When the same experimental protocol was performed by using LPS as the stimulus, a similar degree of suppression of IL-1β expression was seen with HC and PTF to 77.6% ± 1.13% (SEM) of the maximum response with 1 μg of HC per ml and 80.1% ± 10% (SEM) of the maximum response with 1,000 μg of PTF per ml. A81 was not evaluated in LPS-stimulated cultures. When IDM was incubated with AB-stimulated MNCs, variable amounts of enhancement and suppression of IL-1β expression were noted. However, analysis of the grouped data (n = 7) revealed that there was no significant difference from data for the control at any of the IDM doses that were used. MEP and DPH had no effect (data not shown). The administration of HC and PTF to MNCs at selected time intervals before AB exposure had no significant effect on IL-1β expression in comparison with the effect of simultaneous administration (Fig. 3).

**DISCUSSION**

Fever accompanying AB administration is a common therapeutic problem which is often treated with a variety of premedications. However, the pathogenesis of AB-induced fever has not been conclusively elucidated. In contrast, the exogenous pyrogen endotoxin has been used extensively to study the cellular and biochemical mediators of fever. Endotoxin has been shown both in vitro and in vivo to cause the expression of a number of proinflammatory cytokines, including IL-1α and TNF. These cytokines are critical mediators of fever, but the threshold concentrations in serum necessary for inducing fever in humans have not been defined (19). Several observations link these same cytokines with the pathogenesis of AB-induced fever. For example, AB infusion results in a fever curve which simulates cytokine infusion (3, 4, 6, 13, 21). When AB is administered to endotoxin-tolerant rabbits, the fever is attenuated (1). Also, recent investigations with human mononuclear leukocytes and murine macrophages have shown that, in vitro, AB induces the production of prostaglandin E2 (9) and TNF (2). In both of the studies (2, 9), AB was incubated with the target cells for 6 to 24 h, which is outside the window of 60 to 180 min when AB-induced fever is usually observed in humans (3). The initial goal of our studies was to evaluate whether AB could induce IL-1β expression in an in vitro scheme which more closely corresponds to clinical experience. Furthermore, we also used this in vitro model to evaluate the effect of a variety of premedications in down-regulating IL-1β expression by AB-stimulated MNCs.

After a 2-h incubation with human MNCs, AB and LPS produced similar IL-1β dose-response curves, although LPS was severalfold more potent than AB when they were compared at equivalent concentrations. AB concentrations of 1 to 2 μg/ml, which are easily achievable in human serum, resulted in IL-1β expression ranging from 300 to 600 pg/ml. By comparison, similar concentrations of IL-1β were noted when LPS was incubated with MNCs at concentrations of 0.0001 to 0.0005 μg/ml. These findings confirm and extend the work of Gelfand and coauthors (8). In their preliminary studies, AB (2.5 μg/ml) was incubated with human MNCs for 2 h prior to assay for IL-1β. Forty percent of their experiments revealed IL-1β expression at a level of 250 to 310 pg/ml per ml.

We also performed a limited number of experiments to evaluate the effect of adding suboptimal concentrations of LPS and AB simultaneously in culture. In those studies, no significant additive, synergistic, or antagonistic effect on MNC IL-1β expression was documented. Specifically, the IL-1β expression in cultures stimulated with both agents was not significantly different from that in cultures with the most potent in vitro stimulus by itself. If these agents were working through the same or linked cell membrane receptor, one could speculate an additive or antagonistic effect as a
characteristic of similar ligands. Synergism might be speculated if the agents acted at two different receptors or levels of IL-1β expression. Further studies are necessary to clarify the mechanism by which IL-1β expression is stimulated by AB.

The use of premedication before treatment with AB is controversial. Many clinicians consider the use of premedications to be essential for the initial AB therapy to be tolerated. Limited studies in humans have reported on the effectiveness of some premedications in preventing AB-induced fever. For instance, ibuprofen and HC have been shown to be significantly better than placebo in preventing fever associated with AB infusion (9, 27). We used our in vitro system to systematically evaluate the effects of a number of agents, at physiologically achievable concentrations, on modulating AB-induced IL-1β expression. Of those agents that are frequently used as premedications for AB infusion, only HC suppressed IL-1β expression in vitro. Specifically, HC suppressed IL-1β in a dose-related fashion, with a maximal effect noted at HC concentrations of 1 μg/ml or greater. The concentrations of HC in serum range from 0.75 to 2.75 μg/ml after 50- to 200-mg doses are administered parenterally. We used 1% autologous serum in our protocol, because HC is 90% serum protein bound. Thus, the concentrations of unbound HC in our experiments should be representative of the concentrations observed clinically in humans. We did not evaluate the mechanism by which HC exerts its effect on IL-1β expression in our studies. We suspect that the mechanism may be similar to that by which HC inhibits TNF production. Specifically, Han et al. (11) have shown that glucocorticoids strongly inhibit translational derepression of TNF mRNA but only weakly inhibit mRNA accumulation (11). IL-1β gene expression can be detected as early as 30 min after endotoxin exposure, with a peak occurring in 2 to 4 h (15).

Although HC has been shown to be effective in suppressing AB-induced fever and, now, IL-1β production in vitro, the immunosuppressive properties of the drug have led to a debate about its routine use as a premedication. HC can induce transient monocytopenia with a decrease in bacterial and fungal killing by monocytes (20, 22, 23). This may adversely affect patient outcome. If a premedication which prevents AB-induced reactions without immunosuppression could be identified, it would be of potential clinical benefit.

In order to explore alternative agents, we chose to evaluate the methylxanthine derivative PTF and a similar compound (A81) that is under development. Although PTF is marketed as a rheologic agent, it has been shown to exert a number of immunomodulatory effects. Both PTF and A81 inhibited AB-induced IL-1β in a dose-dependent manner. The PTF concentrations that are achievable in the sera of humans after receipt of a 400-mg capsule or solution are similar to the concentrations we investigated in vitro (1,000 μg/ml). Administration of sustained-release tablets of 400 mg results in concentrations in serum of 300 μg/ml (28).

The mechanism by which methylxanthine agents interfere with IL-1β production was not addressed in our studies. It is speculated that down-regulation of cytokine expression might be related to interference with phosphodiesterase and the subsequent conversion of 5′-AMP to cyclic AMP. Additionally, Han and coworkers (11) have shown that PTF suppresses LPS-induced TNF by inhibiting the accumulation of TNF mRNA but that it has little or no effect on mRNA translational derepression. As noted previously, HC suppressed LPS-induced TNF through its major effect on the translational derepression of TNF mRNA. Whether these same mechanisms of action are operative for PTF inhibition of IL-1β in our system will require further study. These data also support the fact that PTF and HC together may suppress IL-1β more than either agent alone does. We did not test this in our system.

Nonsteroidal anti-inflammatory agents, including IDM and ibuprofen (9), have also been used as premedications to ameliorate AB-induced fever. However, IDM did not significantly inhibit IL-1β expression in our studies. This is in agreement with the work of Chia and Pollack (2), in which a nonsteroidal anti-inflammatory agent, indomethacin, did not significantly affect cytokine (TNF) release by AB-stimulated murine macrophages. In similar experiments, Hoffman et al. (12) did not show an effect by indomethacin on endotoxin-stimulated cytokine (IL-1, TNF) expression by human MNCs in vitro. Alternatively, IDM has been shown by Endres et al. (7) and Kunkel et al. (17) to actually enhance IL-1β expression in vitro, most likely by decreasing prostaglandin E₂, which acts as a negative feedback on IL-1 expression. However, a recent study by Kassis et al. (14) demonstrated that prostaglandin E₂ increased cyclic AMP and significantly increased secreted IL-1 from MNCs (14). Thus, conflicting data have shown that there is no effect, enhancement, or suppression of cytokine expression by nonsteroidal anti-inflammatory agents. In the previous experiments (7, 14, 17), IDM was cocultured with the stimuli and target cells for 24 h prior to assay for IL-1. This is in contrast to the 2-h incubation used in the studies described here. The shorter incubation times in our studies may not be associated with suppression of prostaglandin E₂ that was sufficient enough to affect IL-1β expression. Preliminary data from our laboratory indicate that prostaglandin E₂ production by AB-stimulated MNCs is inhibited significantly more when IDM is coincubated for 24 h prior to supernatant harvest and assay than when it is coincubated for 2 h (data not shown). Although IDM and other nonsteroidal anti-inflammatory agents have proved to be effective in ameliorating AB-induced fever (9), our data support the fact that the physiologic effect on fever occurs after cytokine production. Most likely, these agents exert their antipyrogenic effects in the hypothalamus by decreasing the levels of prostaglandins or other arachidonic acid derivatives that have been shown to be important in the pathogenesis of fever. Nonsteroidal anti-inflammatory agents have been shown to decrease cytokine-induced prostaglandin E₂ expression (5, 16, 18).

In summary, AB stimulated MNC IL-1β production in a dose-dependent manner. HC, PTF, and A81 each significantly inhibited AB-induced IL-1β expression, while IDM, DPH, and MEP had no significant effect at the doses tested. The responses noted in vitro lead to our conclusion that HC, PTF, and A81 inhibition of IL-1β expression could result in the reduction of AB-related febrile reactions. This conclusion is supported by the results of in vitro experiments, which have demonstrated that both corticosteroids (23, 24) and the methylxanthine derivatives (25, 26) can affect cytokine release and inflammatory responses. Further work is necessary to evaluate the mechanism and efficacy of these agents in preventing AB-induced fever and chills.

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