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Amphotericin B Both Inhibits and Enhances T-Cell Proliferation: Inhibitory Effect Is Mediated Through H_2O_2 Production via Cyclooxygenase Pathway by Macrophages

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Abstract Amphotericin B (AmB) has been shown to have both immunosuppressive and -enhancing effects, making its precise nature of action enigmatic. In the present study, we found that AmB inhibited concanavalin A (Con A)-induced T cell proliferation if added within first 30 min of stimulation, after which inhibition began to diminish rapidly. However, AmB did not inhibit T-cell proliferation induced by a combination of PMA and ionomycin. AmB inhibition of Con A-induced proliferation was completely overcome by cyclooxygenase inhibitor ibuprofen ([α-methyl-4-(isobutyl)phenylacetic acid]) and H_2O_2 scavenger catalase. In fact, in the presence of ibuprofen and catalase, AmB enhanced, instead of suppressing, Con A-induced proliferation in a dose-dependent way. The effect of catalase was limited to the removal of extracellular H_2O_2 only, as the enzyme did not enter the cells. AmB stimulated H_2O_2 production by macrophages, but not by a lymphocyte population, which was inhibited by ibuprofen. Our T-cell preparation contained about 3% macrophages, and AmB inhibition of proliferation was further pronounced by increasing the macrophage number by as little as 1%. Finally, AmB inhibition of Con A-induced T-cell proliferation was completely overcome by 2-mercaptoethanol. On the basis of these results, we suggest that AmB stimulates H_2O_2 production by macrophages through the activation of the cyclooxygenase pathway of arachidonate metabolism. H_2O_2 then inhibits Con A-induced T-cell proliferation by interfering with an early step of the T-cell receptor signaling pathway through the oxidative modification of some signaling proteins. Our results also show that AmB enhances T-cell proliferation, which can be seen only after blocking its inhibitory effect. J. Cell. Biochem. 77: 361–371, 2000. © 2000 Wiley-Liss, Inc.

Key words: amphotericin B; cyclooxygenase; catalase; H_2O_2; immunomodulation; T cells

Amphotericin B (AmB), a polyene antibiotic, is used to treat the deep-seated fungal infection in human and control fungal contamination in animal cell culture. The mode of antifungal action of amphotericin B involves its binding to plasma membrane sterol, in which it makes holes. The therapeutic value of the drug is based on its higher affinity for fungal sterol (ergosterol) compared with animal sterol (cholesterol) [Brajtburg and Bolard, 1996]. Nevertheless, the drug has been shown to cause kidney toxicity by mechanisms other than sterol-binding [Brouhard and Baetz-Greenwalt, 1992; Sabra and Branch, 1992; Zager et al., 1992; Haag-Weber and Horl, 1994]. In addition, AmB has been shown to inhibit erythropoiesis, to cause chill and fever, to inhibit DNA synthesis in vitro, and to cause lung injury in human and rodents [Koeffler and Golde, 1977; Kuwano et al., 1981; Gigliotti et al., 1987; McDonnell et al., 1988].

Several studies have reported both the immunoenhancing and immunosuppressive properties of AmB. Thus, the antibiotic has been shown to both stimulate and suppress macrophage, B-cell, and T-cell activity in culture [Hammarstrom and Smith, 1976; Walls and Kay, 1982; Mehta et al., 1985; Boggs et al., 1991; Wilson et al., 1991; Schindler et al., 1993] and to inhibit induced, but not spontaneous, natural killer (NK) cell activity in vitro

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[Hauser and Remington, 1983]. A differential effect of AmB was observed with human peripheral blood lymphocytes (PBL) and tonsil lymphocytes (TL) [Seay and Inman, 1982]. The TL response was inhibited at any dose of the AmB. However, the response of PBL to Concanavalin A (Con A) was enhanced at lower doses but suppressed at higher doses of the drug. Such a bidirectional effect of the antibiotic was also seen in murine L cells [Brajtburg et al., 1984]. It has been shown that mice strains in which AmB is an immunoadjuvant has a higher level of catalase activity in spleen cells, macrophages, and hepatocytes, as compared with mice in which the drug is immunosuppressive [Stein et al., 1987; Wolf et al., 1991]. Interestingly, it has been reported that a nontoxic thiol derivative of AmB induced a strong proliferative and IgG secretion response under conditions in which the parent AmB is toxic [Sarthou et al., 1986]. While all these reports indicate an immunomodulatory effect of AmB, its precise nature of effect and precise mechanism of action on the immune system is unknown.

In the present study, we investigated the nature of the modulatory action of AmB on murine T cells. We found that the antibiotic exerted both the inhibitory and enhancing effect on Con A-induced T-cell proliferation. The inhibitory effect was exerted at an early step of T-cell receptor signaling pathway through the stimulation of H_2O_2 production via the cyclooxygenase pathway of arachidonate metabolism by macrophages. The enhancing effect of AmB on T-cell proliferation can be seen after blocking its inhibitory effect.

MATERIALS AND METHODS

Reagents

Amphotericin B was purchased from Sarabhai Chemicals (Baroda, India). Concanavalin A was purchased from Pharmacia AB (Uppsala, Sweden). Horseradish peroxidase (HRP) was purchased from Boehringer-Mannheim (Indianapolis, IN). H_2O_2 and 2-mercaptoethanol (2-ME) were purchased from E. Merck Limited (Mumbai, India). Ionomycin was purchased from Calbiochem-Novabiochem International (San Diego, CA). Catalase (hydrogen peroxide: hydrogen peroxide oxidoreductase; EC 1.11.1.6), L-glutamine, Hank's balanced salt solution (HBSS), MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; thiazolyl blue]), o-phenylenediamine (OPD), and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO). Ibuprofen ([α-methyl-4-(isobutyl)phenylacetic acid]) was purchased from Cipla Pharmaceutical Company, India. Roswell Park Memorial Institute-1640 (RPMI-1640) medium was from HiMedia Laboratories Limited (Mumbai, India). [^{3}H]-thymidine (spec act 18,000 mCi/mmole) was from Bhabha Atomic Research Center (Mumbai, India). Fetal bovine serum (FBS) was purchased from Gibco-BRL Life Technologies (Grand Island, NY).

Cells and Culture Medium

T cells and macrophages were isolated from the spleen of 6- to 8-week-old Balb/c mice. Unless otherwise mentioned, isolated T cells and macrophages were maintained and cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 0.1 mg/ml streptomycin, and 0.2 mg/ml gentamycin (complete medium) at 37°C in a humidified atmosphere of 5% CO_2 in air. Single-cell suspension of splenocytes was prepared by gently crushing the spleen on a wire mesh in RPMI-1640. 1×10^8 splenocytes were cultured in complete medium for 2 h in a 90-mm culture dish. The nonadherent cells (lymphocytes) were removed gently, and T cells were isolated from this by nylon wool column method as described before [Chakrabarti et al., 1995]. The adherent cells (macrophages) were washed gently with RPMI-1640 and dislodged from the culture dish by gentle stream of RPMI-1640 through an 18-gauge needle from a 10-ml syringe.

Cell Proliferation Assay

Proliferation of T cells was measured by the incorporation of [^{3}H]thymidine in the replicating DNA. T cells (1×10^5 cells/well in 0.1 ml) were stimulated with Con A or with a combination of PMA and ionomycin in the presence or absence of various agents in round-bottom 96-well plates. The cells were pulsed with [^{3}H]thymidine (1 μCi/ml) at 24 h and harvested with a PHD cell harvester at 60 h of culture; [^{3}H]thymidine incorporation was measured by a liquid scintillation counter. The effect of various agents on proliferation was expressed as

Percentage of control proliferation

= 100 × proliferation in the presence of the agent/proliferation in the absence of the agent (control proliferation)

Determination of Cell Viability

The viability of T cells, after treatment with various agents, was measured by MTT assay [Mosmann, 1983]. T cells were washed and incubated with 2.5 mg/ml MTT in complete medium for 4 h at 37°C in 5% CO_2 incubator. After washing the cells with normal saline, the formazone crystals formed were solubilized in acidic isopropanol (with 0.04 N HCl). The absorbance of the solution was measured at 492 nm in E_{max} automated enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, Palo Alto, CA).

Measurement of Amphotericin B-Induced H_2O_2 Production by Splenocytes

Measurement of H_2O_2 secreted in the medium by splenocytes was based on the HRP-catalyzed reduction of H_2O_2 in the presence of o-phenylenediamine (OPD); 5×10^6 total splenocytes, macrophages (adherent cells), total lymphocyte, and T-cell-enriched fractions were stimulated with 2 μg/ml AmB in Hank's balanced salt solution (HBSS) at 37°C for the stipulated period of times. After stimulation, cells were pelleted out and the supernatant collected; 0.05 ml of standard H_2O_2 or the samples were added to the equal volume of 0.2 M citrate-phosphate buffer (pH 5) containing HRP and OPD at the final concentration of 7.5×10^{-3} U/ml and 1 mg/ml, respectively. After incubation at 37°C for 15 min in dark, the enzymatic reaction was stopped by adding 0.1 ml of 3 N HCl; absorbance was read at 492 nm λ (A_{492}) in E_{max} microplate reader. The H_2O_2 concentrations of the samples were obtained from the curve of A_{492} versus known H_2O_2 concentration (4–500 μ). The actual concentration in the original supernatant was obtained by multiplying the H_2O_2 concentration in the diluted samples by the dilution factors. In some experiments, splenocytes were preincubated with 0.5 mg/ml ibuprofen for 30 min at 37°C before stimulation with AmB.

Determination of Catalase Entry into the Splenocytes

The entry of catalase, added extracellularly to the splenocytes, was determined on the basis of catalase-catalyzed H_2O_2 degradation, measured based on the reduction of absorbance of H_2O_2 at 240 nm [Aebi, 1994]. In brief, 5×10^6 splenocytes in 0.2 ml HBSS were incubated with 5×10^4 U/ml catalase for 4 h at 37°C and 4°C. After incubation, the cells were washed thoroughly and resuspended in 0.1 ml HBSS. Any residual amount of extracellular catalase was degraded by proteinase K (1 mg/ml) treatment for 15 min at 37°C. The cells were washed thoroughly to remove proteinase K, resuspended in 0.25 ml HBSS containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and sonicated at 40 KHz in four short bursts (each 10 s) in ice cool condition. The samples were allowed to cool for 30 s between each burst of sonication. Cell debris from the samples was removed by hard spin and clear supernatant collected; 0.2 ml cell lysate or a known amount of catalase (2–1,000 U/ml) was incubated with 16 mM H_2O_2 in 1 ml reaction volume in a quartz cuvette for 30 s, and the reduction of H_2O_2 absorbance at 240 nm λ (A_{240}) was read continuously throughout the 30-s period.

RESULTS

AmB Inhibits Con A-Induced T-Cell Proliferation Dose- and Time-Dependently

The T cells are bestowed with clonospecific antigen receptor called T-cell receptor (TCR) in close physical association with a multimeric protein complex the CD3 complex [Truneh et al., 1985; Hadden, 1988]. This complex is essential to deliver the activation signal inside the cell after occupancy of the TCR by antigen. The action of antigen on T cells can be mimicked by antibodies to the TCR/CD3 complex or plant lectins, such as Con A.

To determine the effect of AmB on T-cell proliferation stimulated through TCR/CD3 complex, freshly isolated T cells were stimulated with Con A in the presence or absence of the drug. AmB caused dose-dependent inhibition of Con A-stimulated proliferation of T cells, with about 50% inhibition at 1 μg/ml of the drug (Fig. 1A). A maximum of about 70% inhibition, without any change in cell viability, was obtained with 2 μg/ml AmB. Therefore, in all subsequent experiments, a maximum dose

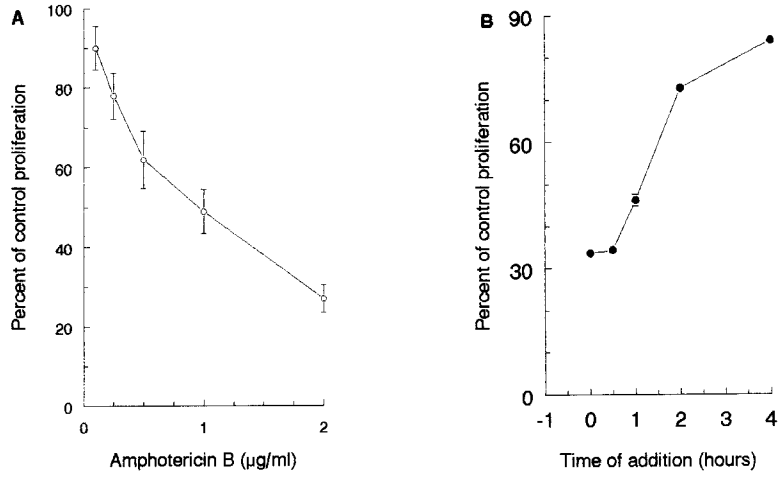

Fig. 1. Inhibition of Concanavalin A (Con A)-induced T-cell proliferation by amphotericin B (AmB). 1×10^5 T cells were stimulated with 10 μg/ml Con A in 96-well plates for 60 h and DNA synthesis measured based on [^{3}H]thymidine incorporation as under Materials and Methods. **A:** Various doses of AmB added to the cells at the beginning of stimulation. **B:** 2 μg/ml AmB added to the cells at different time of stimulation. Percentage (%) of control proliferation was calculated as 100 × proliferation in the presence of the agent/proliferation in the absence of the agent (control proliferation). The results are the mean ± SEM of three experiments. Unstimulated and stimulated control [^{3}H]thymidine incorporation (cpm ± SEM/10^5 cells/well) in **A:** 338 ± 82 and 31824 ± 4267; in **B:** 229 ± 36 and 27900 ± 277.

of 2 μg/ml AmB was used. The inhibitory effect of AmB was further studied through its addition to the cells at different times of stimulation. As shown in Figure 1B, AmB inhibited Con A-induced proliferation of T cells in a time-dependent manner. The addition of AmB within first 30 min of stimulation resulted in about 70% inhibition of proliferation. However, the inhibitory effect of AmB began to diminish by 1 h (~50% inhibition), was substantially reduced by 2 h (~27% inhibition), and almost completely diminished by 4 h (~16% inhibition) of stimulation. These results clearly showed that AmB inhibited Con A-induced T-cell proliferation at an early step of TCR signaling pathway.

AmB Did Not Inhibit PMA/Ionomycin-Induced Proliferation of T Cells

The principal intracellular signaling events, which follow the interaction of antigen/mitogen with TCR/CD3 complex, are the activation of protein kinase C (PKC) and a rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [Truneh et al., 1985; Hadden, 1988]. These two events initiate the entire cascade of events that constitute T-cell activation. Therefore, T cells can be stimulated to proliferate by directly activating PKC with a phorbol ester and increasing $[Ca^{2+}]_i$ with a Ca^{2+} ionophore without the requirement of stimulation through TCR/CD3 complex [Truneh et al., 1985; Berry et al., 1990]. Thus, to determine the site and specificity of AmB action on Con A-induced T-cell proliferation. we explored whether AmB can inhibit T-cell proliferation stimulated with a combination of PMA and ionomycin. PMA or ionomycin alone do not induce any proliferation (data not shown). A combination of both induced a huge proliferation of T cells, which was not affected by even the highest effective concentration of AmB (2 μg/ml) (Fig. 2), indicating that AmB specifically inhibited T-cell proliferation stimulated through TCR/CD3 complex.

Effect of Ibuprofen on the Antiproliferative Effect of AmB

AmB has been shown to stimulate prostaglandin E_2 (PGE_2) synthesis in human and murine mononuclear cells [Gigliotti et al., 1987], and PGE_2 has been reported to inhibit T-cell activation [Anastassiou et al., 1992]. Thus, we explored the possible involvement of PGE_2 in mediating AmB effect on T-cell proliferation, by examining the effect of ibuprofen on this process. Ibuprofen is the specific inhibitor of cyclooxygenase, a key rate-limiting enzyme involved in the metabolism of arachidonate to prostaglandins [Smith and DeWitt, 1996]. Our results showed that ibuprofen overcome the inhibitory effect of AmB on Con A-induced T-cell proliferation dose-dependently, with almost complete overcome at 0.1 mg/ml (Fig. 3A). Interestingly, ibuprofen also led to 43% enhancement of the proliferation over the control at the concentration of 0.5 mg/ml (Fig. 3A). This enhancement was observed only in the presence of AmB, as ibuprofen alone did not affect Con A-induced proliferation at the concentrations used in this study (see Fig. 7).

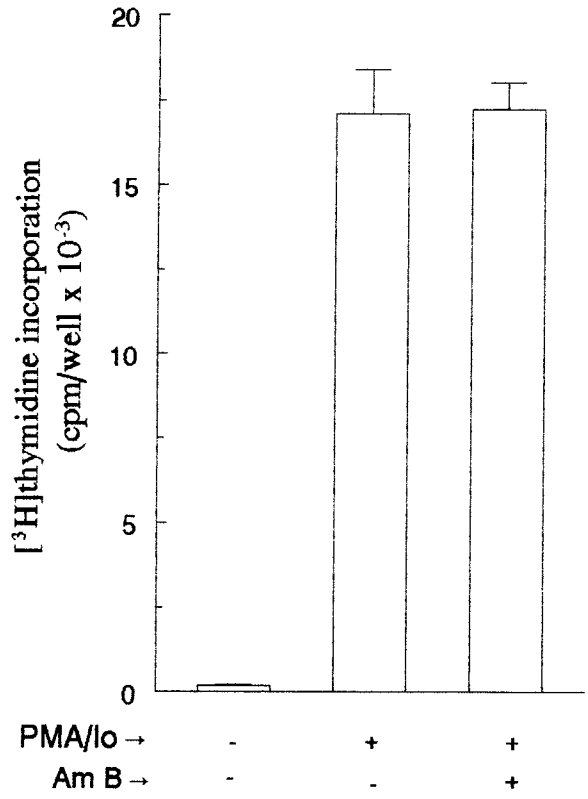

Fig. 2. Amphotericin B (AmB) did not inhibit PMA/ionomycin-induced proliferation of T cells. T cells were stimulated to proliferate as in Fig. 1 with a combination of 0.25 nM PMA and 2 μ ionomycin in the presence or absence of 2 μg/ml AmB. Results are the mean ± SEM of three experiments.

Effect of Catalase on the Antiproliferative Effect of AmB

In addition to stimulating PGE_2 production, AmB has been shown to stimulate or enhance the production of superoxide anion (O_2^-) in murine macrophages [Stein et al., 1987; Wilson et al., 1991; Wolf et al., 1991] and to involve H_2O_2 production, inducing injury to lung tissue [McDonnell et al., 1988]. Further, the immune cells from mice in which AmB is either an immunoadjuvant or is ineffective have high catalase activity [Stein et al., 1987], prompting us to explore the possible involvement of H_2O_2 in mediating the antiproliferative effect of AmB, by examining the effect of exogenously added catalase in this process. We found that catalase overcomes the inhibitory effect of AmB on Con A-induced T-cell proliferation dose-dependently, with complete overcome at 250 U/ml (Fig. 3B). Like ibuprofen, catalase also showed the tendency to enhance proliferation, with approximately 33% enhancement over control at the dose of 1,000 U/ml obtained (Fig. 3B). The enhancing effect of catalase on Con A-stimulated T-cell proliferation was observed only in the presence of AmB, as catalase alone did not affect Con A-induced proliferation (see Fig. 7).

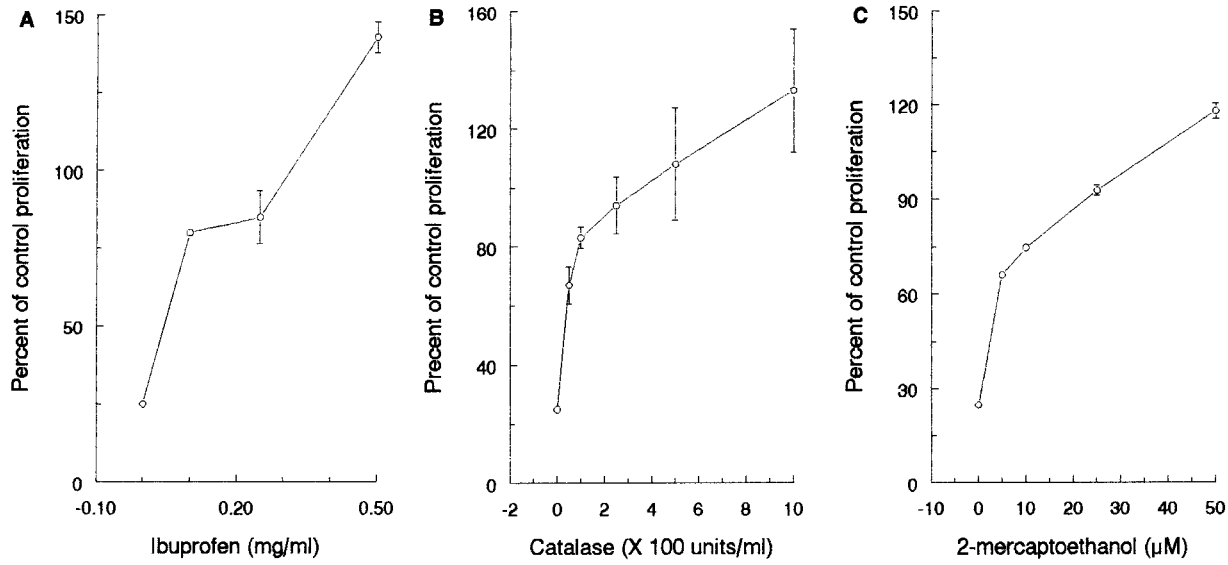

Fig. 3. Ibuprofen, catalase, and 2-mercaptoethanol overcome the inhibitory effect of amphotericin B (AmB) on Concanavalin A (Con A)-induced T-cell proliferation. T cells were stimulated to proliferate with Con A as in Fig. 1 in the presence or absence of 2.0 μg/ml AmB. Various concentrations of Ibuprofen (**A:** n = 3), catalase (**B:** n = 4) and 2-mercaptoethanol (**C:** n = 4), were added to the cells at the beginning of stimulation. Unstimulated and stimulated control [^{3}H]thymidine incorporation in **A:** 149 ± 24 and 24261 ± 3161; **B:** 232 ± 58 and 21548 ± 448; **C:** 154 ± 19 and 22853 ± 1757.

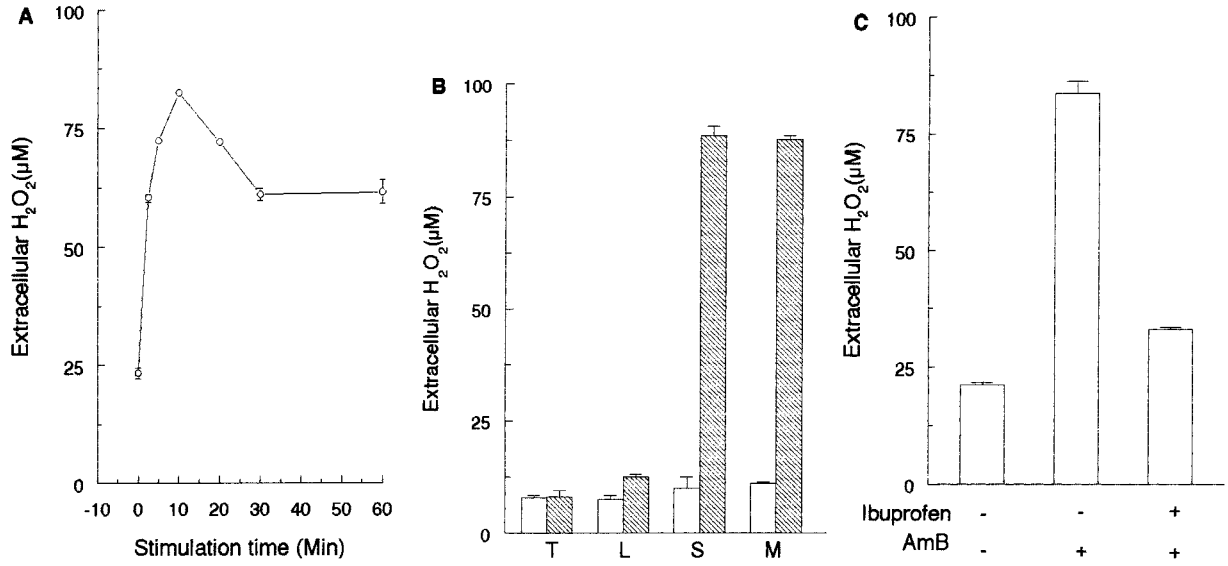

Fig. 4. Amphotericin B (AmB) stimulates H_2O_2 production by splenocytes through an ibuprofen-sensitive mechanism. **A:** 5×10^6 splenocytes were stimulated with 2 μg/ml AmB for various time periods (n = 3). **B:** 5×10^6 cells of total splenocytes (S), total lymphocytes (L), T cells (T), and plastic adherent cell (macrophages, M) were stimulated with (hatched bar) and without (open bar) 2 μg/ml AmB for 10 min (n = 4). **C:** 5×10^6 splenocytes were sham-treated or treated for 30 min with 0.5 mg/ml ibuprofen before stimulation with 2 μg/ml AmB (n = 3). After incubation for the stipulated time periods, cells were spun down and concentration of H_2O_2 in the supernatant measured as described under Material and Methods.

AmB Stimulates H_2O_2 Production by Splenocytes Through an Ibuprofen-Sensitive Mechanism

As catalase reversed the antiproliferative effect of AmB, we explored whether the antibiotic induced any H_2O_2 production by spleen cells. Thus, splenocytes were stimulated with 2 μg/ml AmB for various times and the secreted H_2O_2 in the medium measured. AmB stimulated time-dependent production of H_2O_2 by the splenocytes (Fig. 4A). The level of H_2O_2 secreted by the unstimulated cells in the medium was about 23 μM. Increased levels of H_2O_2 after AmB stimulation were detected as early as 2.5 min (~60 μM), attaining maximum levels (~82 μM) by 10 min of stimulation. The H_2O_2 level then declined slowly to a level of 61 μM by 30 min and that level was maintained up to 60 min of stimulation. To ascertain the cell type involved, we measured the AmB-stimulated H_2O_2 production by total splenocyte, total lymphocyte, T-cell and macrophage (plastic adherent cells) fractions of splenocytes. We failed to detect any H_2O_2 secretion by T cells (Fig. 4B). Total lymphocytes showed some increase in H_2O_2 secretion in response to AmB. However, unfractionated splenocytes and macrophages showed a dramatic increase in H_2O_2 production in response to AmB. These results showed that AmB stimulates H_2O_2 production not by lymphocytes, but by nonlymphocytic leukocytes, which are mainly macrophages among the splenocytes.

As both catalase and ibuprofen completely overcome the antiproliferative effect of AmB, we explored whether ibuprofen can inhibit AmB-induced H_2O_2 production. Our results showed that 30-min prior treatment of splenocytes with 0.5 mg/ml ibuprofen resulted in a 80% inhibition of AmB-induced H_2O_2 production (Fig. 4C). This result indicates that AmB stimulates H_2O_2 production through the cyclooxygenase pathway of arachidonate metabolism.

Extracellularly Added Catalase Did Not Enter the Splenocytes

We sought to determine whether H_2O_2 directly inhibits T-cell proliferation or generates some compounds inside the originator cells, which in turn affect T-cell proliferation. This was tested by examining whether extracellular addition of catalase can degrade intracellular H_2O_2 also by entering into the cells, as in some

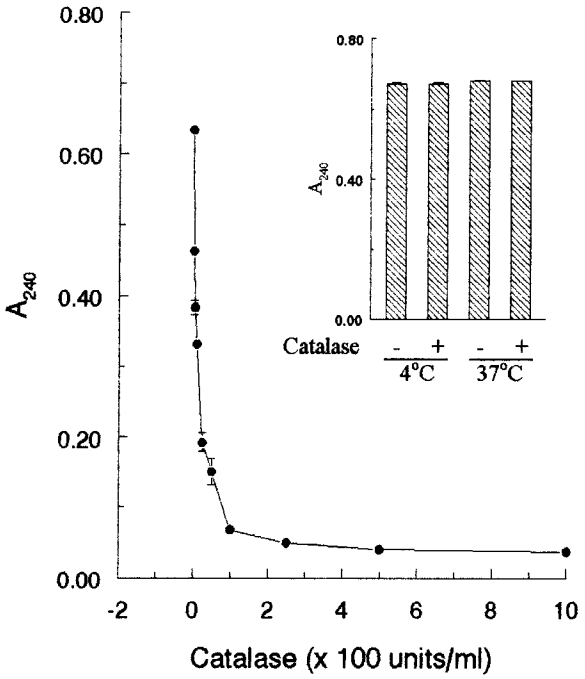

Fig. 5. Determination of catalase entry into the splenocytes. 5×10^6 splenocytes were incubated for 4 h at 4°C and 37°C in the presence or absence of 5×10^4 U/ml catalase in 0.2 ml complete medium. Cells were washed, treated with proteinase K, washed again, and sonicated in ice cooling condition in 0.25 ml Hank's balanced salt solution (HBSS) containing 1 mM PMSF. 0.2 ml cell lysate (inset) and the known amount of catalase (2–1,000 U/ml final concentrations) was incubated with 16 mM H_2O_2 in 1 ml quartz cuvette for 30 s and absorbance at 240 nm (A_{240}) were read.

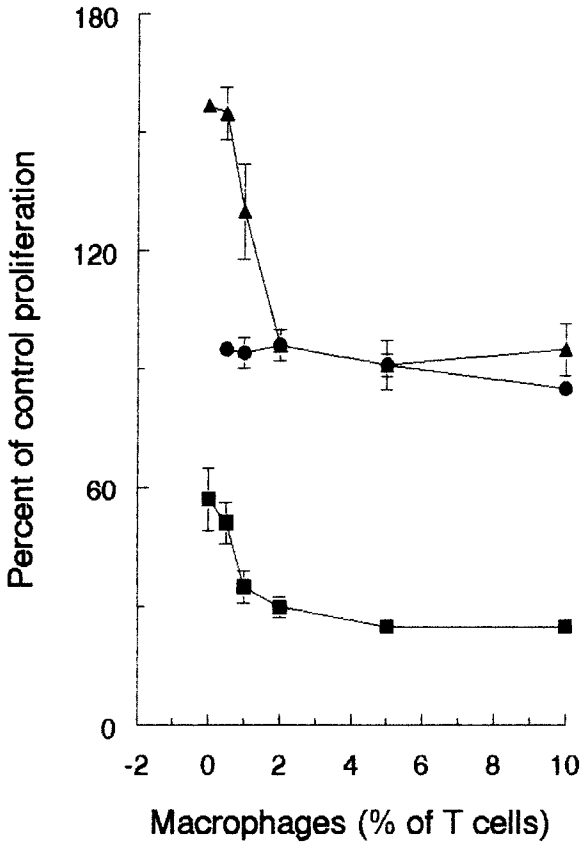

Fig. 6. Effect of macrophages on Concanavalin A (Con A)-induced T-cell proliferation. T cells were stimulated to proliferate with Con A as in Fig. 1, in the absence (solid circle) or presence of 0.5 μg/ml amphotericin B (AmB) (solid square) or 0.5 μg/ml amphotericin B (AmB) + 0.5 mg/ml ibuprofen (solid triangle). Various amounts of macrophage were added to all at the beginning of stimulation. Results are the mean ± SEM of three experiments. Unstimulated and stimulated control [^{3}H]thymidine incorporation are 372 ± 45 and 39202 ± 1731, respectively.

cells catalase can enter the cell [Sundaresan et al., 1995]. Thus, splenocytes were incubated with 5×10^4 U/ml catalase for 4 h and washed; any trace amounts of extracellular catalase were degraded by proteinase K treatment; cells were sonicated and catalase activity measured in the cell lysate. Our results show that we can detect as low as 2 U/ml catalase, which reduced A_{240} of 16 mM H_2O_2 from 0.6 to 0.4 in 30 s (Fig. 5). The maximum reduction in A_{240} (0.6–0.07) was obtained with 100 U/ml catalase in 30 s. However, the cell lysates did not have any detectable catalase activity, as they did not cause any reduction in A_{240} of 16 mM H_2O_2, indicating that catalase can not enter into the splenocytes (Fig. 5, inset).

Macrophages Mediate the Inhibitory Effect of AmB

Freshly isolated T-cell preparations are always contaminated with some non-T cells. In the present study, the T-cell preparation contained about 3% of macrophages. To establish that the contaminating amount of macrophages present in the T-cell preparation mediated the inhibitory effect of AmB through H_2O_2 production, we examined the effect of macrophages on the antiproliferative effect of a submaximal dose of AmB. Thus, T cells were stimulated with Con A in the presence or absence of 0.5 μg/ml AmB with or without ibuprofen, to which different amounts of macrophages (percentage of total cells in the culture) were added. The results presented in Figure 6 show that without any added macrophages, AmB leads to only about 40% inhibition in Con A-induced T-cell proliferation. However, the addition of macrophages increased the extent of inhibition dose-dependently (e.g., from 40% to

70% inhibition with the addition of just 2% macrophages). In the presence of 0.5 mg/ml ibuprofen, AmB caused the expected enhancement (~50% over control) of proliferation, which was reduced to control level, but not below that by the addition of macrophages. Macrophages added in the absence of AmB or ibuprofen did not affect Con A-induced proliferation.

2-Mercaptoethanol Overcomes the Antiproliferative Effect of AmB

H_2O_2 has been shown to cause oxidation of methionine and cysteine residues of many proteins, rendering them inactive; this process can be reversed by a reducing agent, such as a reducing enzyme or dithiothreitol, which can substitute for the biological reducing system [Brot et al., 1981; Abrams et al., 1981; Hecht and Zick, 1992; Caselli et al., 1998]. For example, H_2O_2 has been shown to inhibit protein tyrosine phosphatase (PTP) activity by oxidizing the conserved cysteine residues essential for its catalytic activity, which was reversed by dithiothreitol [Hecht and Zick, 1992; Caselli et al., 1998]. Thus, in an attempt to decipher the mechanism whereby H_2O_2 mediated the effect of AmB, we examined whether the reducing agent 2-mercaptoethanol (2-ME) can overcome the antiproliferative effect of AmB. Our results showed that 2-ME overcame the suppressive effect of AmB on Con A-induced T-cell proliferation dose-dependently, with a complete reversal of the effect at 25 μM 2-ME (Fig. 3C), indicating that H_2O_2 may affect T-cell activation through the oxidation of critical amino acids of some critical proteins.

AmB Enhanced Con A-Induced T-Cell Proliferation in the Presence of Ibuprofen and Catalase

As ibuprofen and catalase showed the tendency to enhance proliferation in the presence of AmB, we explored whether AmB can enhance, rather than inhibit, the proliferation dose-dependently, in the presence of these agents. Thus, T cells were stimulated with Con A, in the presence of various concentrations of AmB in combination with 1,000 U/ml catalase or 0.5 mg/ml ibuprofen. The results are presented in Figure 7. Ibuprofen or catalase alone (0 dose of AmB) did not affect Con A-induced T-cell proliferation. In the presence of ibuprofen, AmB was found to enhance, rather than suppress, Con A-induced T-cell proliferation dose-dependently. A maximum enhancement of about 80% over control was obtained with 1μg/ml of AmB. With increasing doses of AmB, the enhancement was reduced, with only 17% enhancement observed with 2 μg/ml AmB. Similarly, in the presence of catalase, AmB enhanced Con A-induced T-cell proliferation dose-dependently, with maximum enhancement of about 50% over control at 1 μg/ml AmB. This result showed that AmB enhances T-cell proliferation, which can be revealed only by neutralizing its inhibitory effect.

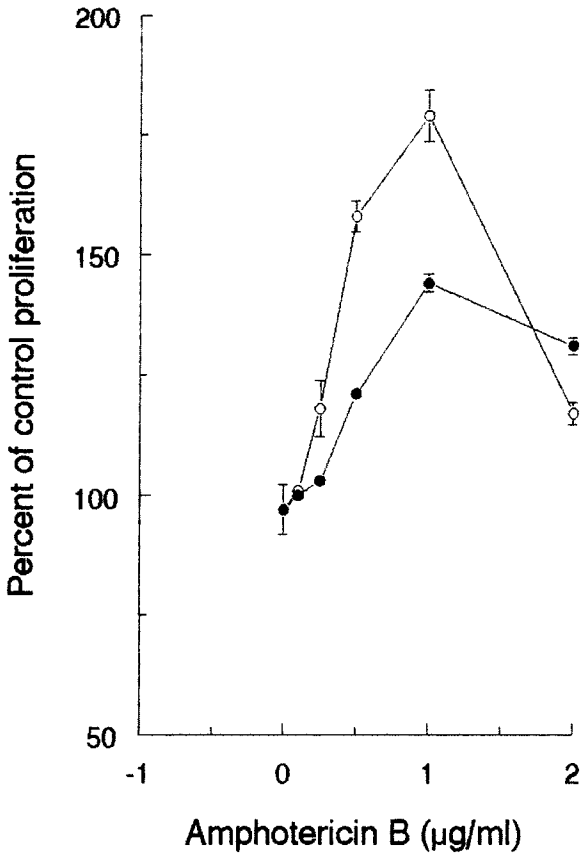

Fig. 7. Amphotericin B (AmB) enhances Concanavalin A (Con A)-induced T-cell proliferation in the presence of ibuprofen and catalase. T cells were stimulated to proliferate with Con A in the presence or absence of 0.5 mg/ml ibuprofen (open circle) or 1,000 U/ml catalase (solid circle) and various concentrations of AmB. The results are the mean ± SEM of three experiments. Unstimulated and stimulated control [^{3}H]thymidine incorporation is 239 ± 14 and 35989 ± 2599, respectively.

DISCUSSION

AmB has been reported by different workers to have both immunoenhancing and suppres-

sive effects; thus, its precise effect and mechanism of action on immune system remain enigmatic. In the present study, we provided an insight into the mechanism of action of AmB on T-cell activation. We found that AmB blocked the Con A-induced T-cell proliferation dose-dependently without affecting cell viability. The inhibition is obtained if AmB is added within the first 30 min of stimulation, after which inhibition begins to diminish. By contrast, AmB failed to affect T-cell proliferation stimulated by directly activating PKC and increasing $[Ca^{2+}]_i$ by PMA and ionomycin, respectively. These results clearly showed that AmB inhibited T-cell activation, specifically by blocking an early step of the TCR signaling pathway proximal to PKC activation and to increased $[Ca^{2+}]_i$.

In the present study, we found that ibuprofen, a specific inhibitor of cyclooxygenase [Smith and DeWitt, 1996], completely overcome the inhibitory effect of AmB, showing that AmB exerted the inhibitory effect through activation of the cyclooxygenase pathway of arachidonate metabolism. In addition, exogenous catalase, a highly specific superfast kinetic H_2O_2 degrading enzyme, completely overcame the AmB inhibition of Con A-induced T-cell proliferation. As a complement to this finding, we found that AmB stimulated H_2O_2 production by splenocytes time-dependently. These results clearly showed that AmB inhibited T-cell proliferation through H_2O_2 production. Since catalase cannot enter the splenocyte, the effect of catalase must be limited to the degradation of extracellular H_2O_2. This finding suggests that it is the extracellularly secreted H_2O_2 that affected T-cell proliferation.

Our results have clearly shown that AmB inhibited T-cell proliferation through both activation of the cyclooxygenase pathway and H_2O_2 production. However, both cyclooxygenase inhibitor and H_2O_2 scavenger completely overcomes the effect of AmB. This finding indicates that activation of the cyclooxygenase pathway and H_2O_2 production are interlinked. If both were independently involved, a partial overcome would have been observed with either catalase or ibuprofen. To resolve this issue, we examined the effect of ibuprofen on AmB-induced H_2O_2 production by splenocytes. Our results showed that ibuprofen inhibited AmB-induced H_2O_2 production by splenocytes. This clearly showed that H_2O_2 is produced as a result of the activation of cyclooxygenase pathway of arachidonate metabolism by AmB. This finding is supported by the reports showing that aspirin, a cyclooxygenase inhibitor [Smith and DeWitt, 1996], inhibits H_2O_2 production by granulocytes [Haynes et al., 1993]. Classically, in macrophages, H_2O_2 is generated from superoxide anion (O_2^-), which itself is generated from O_2 catalyzed by the membrane NADPH oxidase. However, how cyclooxygenase pathway is linked to H_2O_2 production is unclear. One possibility is that PGE_2 itself is converted to H_2O_2 by β-oxidation in peroxisome, as in rat liver and kidney [Schepers et al., 1988]. Alternatively, other metabolites of cyclooxygenase pathway directly or indirectly result in H_2O_2 production in macrophages, as thromboxane A_2 is reported to stimulate H_2O_2 production by neutrophils [Paterson et al., 1989]. Yet another possibility is that the cyclooxygenase pathway is somehow linked to the activation of NADPH oxidase pathway.

AmB stimulated H_2O_2 production only by the macrophages, and not by the lymphocyte fraction of splenocytes, raising the question of how AmB inhibited proliferation of purified T cells. It is worth noting that purified preparation of fresh T cells is always contaminated at very low level with other cells. Our T-cell preparation contained about 3% macrophages. The amount of H_2O_2 secreted by this much contaminating cells may be below our detection limit, but may be sufficient to attain a local concentration high enough to inhibit the proliferation of neighboring T cells. This local concentration of H_2O_2 must be higher than we found in splenocyte culture medium (~80 μM), because 100 μ H_2O_2 caused only slight inhibition (34 ± 2%, n = 3) of Con A-induced T-cell proliferation without affecting PMA/ionomycin-induced proliferation (only 6 ± 1% inhibition, n = 3). Thus, minimal contamination with macrophages may be sufficient to mediate the AmB effect. In support of this, we found that increasing the amount of macrophage just by 1% by adding isolated macrophages to the culture resulted in a drastic increase in AmB inhibition of Con A-induced proliferation. However, macrophage alone had no effect on Con A-induced proliferation. This result shows that little amount of macrophages in T-cell preparation is sufficient to mediate the inhibitory effect of AmB on T-cell proliferation through H_2O_2 production. Our finding that AmB inhibited T-cell prolifer-

ation through H_2O_2 production by macrophages was reinforced by previous findings that (1) as low as 3% macrophages can inhibit, and indomethacin (inhibit arachidonate metabolism) and catalase can enhance, Con A-induced proliferation of lymphocytes in aged rats [Franklin et al., 1993; Hayek et al., 1994]; and (2) activated macrophages can inhibit fibroblast and lymphocyte proliferation through H_2O_2 production [Metzger et al., 1980, 1986].

Our results have shown that AmB-generated H_2O_2 inhibited T-cell proliferation by blocking TCR signaling through the oxidative inactivation of some signaling proteins located at, or proximal to, phosphoinositide hydrolysis, which leads to PKC activation and an increase in $[Ca^{2+}]_i$. The identity of these proteins and the mode of inhibition are unknown. However, inhibition of tyrosine kinase activation, which is the very first step of TCR signaling, might be the possible mechanism. Two of these tyrosine kinases *lck* and *fyn* have phosphotyrosine at their C-terminal end, keeping them inactive in resting cells [Mustelin and Burn, 1993]. Upon stimulation of cells, PTP activity of the CD45 molecule removes phosphate from these residues, activating lck and fyn tyrosine kinase. Thus, TCR signaling in CD45 mutants is blocked at the tyrosine kinase activation step [Mustelin and Burn, 1993]. We know that H_2O_2 inactivates PTP through the oxidation of cysteine residues [Hecht and Zick, 1992; Caselli et al., 1998]. CD45 PTP is also inactivated by oxidation of cysteine residues [Garcia-Morales et al., 1990]. Thus, it is highly plausible that H_2O_2 blocked TCR signaling by interfering with the activation of lck and fyn tyrosine kinase through the inactivation of CD45 PTP. In addition, H_2O_2 may interfere with tyrosine kinase activation step by blocking the tyrosine residues of the substrates through the oxidative modification, as H_2O_2 has been shown to oxidize tyrosine to tyrosyl radical [McCormick et al., 1998].

In the present work, we found consistently that the agents that overcome the AmB effect also tend to enhance the proliferation in the presence of the antibiotic, giving rise to the possibility that suppressing the inhibitory effect of AmB might reveal its immunoenhancing effect. To this end, we found that, in the presence of a constant dose of ibuprofen and catalase, AmB enhanced Con A-induced T-cell proliferation dose-dependently. However, with increased doses, the enhancing effect of AmB tends to be reduced. Thus, these results clearly showed that the enhancing and suppressing effects of AmB on T-cell activation are mutually exclusive. When one predominates, the other subsides. Thus, when catalase and ibuprofen concentration is limited (constant), the enhancing effect of AmB tends to be reduced beyond certain concentration. Similarly, when AmB concentration is limited (constant), its suppressive effect tends to disappear and enhancing effect appear beyond certain concentrations of ibuprofen and catalase.

In a nutshell, our work showed that AmB has both suppressing and enhancing effects on T-cell activation, which are mutually exclusive. To exert the inhibitory effect, AmB stimulates macrophages to produce H_2O_2, which in turn inhibits T-cell proliferation by interfering with an early step of TCR signaling pathway proximal to PKC activation and increase in $[Ca^{2+}]_i$.

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