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Suppression of immunological responses in mice by treatment with amphotericin B

A. FERRANTE, B. ROWAN-KELLY & Y. H. THONG *Department of Paediatrics, University of Adelaide, The Adelaide Children's Hospital, North Adelaide*, 5006, *South Australia*

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SUMMARY

The polyene antibiotic amphotericin B (AmB) caused a marked suppression of the cell-mediated immune response in mice. Similar treatment did not affect the humoral antibody response. The immunosuppressive property of the drug was related to its ability to inhibit the manifestation rather than the induction phase of the delayed-type hypersensitivity response.

In vitro AmB suppressed mitogen-induced lymphocyte proliferation. The drug seemed to act at the proliferative phase of the response. Results presented show that the T cell response was much more sensitive to the action of AmB than was the B cell response. During AmB chemotherapy consideration must be given to the immunosuppressive properties of this drug.

INTRODUCTION

Amphotericin B (AmB) is a polyene antibiotic, very useful for the treatment of systemic fungal infections (Bennet, 1974). Conflicting results have been reported with regard to the effect of this drug on the immune response. A single dose of AmB has been shown to enhance both cell-mediated and humoral immune responses in mice (Blanke *et al.*, 1977; Ishikawa, Narimatsu & Saito, 1975), while its presence in culture suppressed mitogen-induced human lymphocyte proliferative responses (Tärnvik & Ånséhn, 1974; Thong & Rowan-Kelley, 1978). The effect of multiple doses of AmB on murine immunological responses has not been studied. In the clinical situation, AmB is administered in multiple doses over many days or weeks. It is important, therefore, to determine the effect of a course of AmB treatment on immunological responses because the enhancement or suppression of such a response may influence the outcome of the infection being treated. This is especially important in the case of AmB because patients with deep mycotic infections are usually already immunocompromised.

MATERIALS AND METHODS

Mice. Male BALB/c mice, 6–8 weeks old, were obtained from the Institute of Medical and Veterinary Science, Adelaide. These were kept in an air-conditioned animal house and allowed free access to food and water.

Amphotericin B (AmB). For *in vivo* studies, the AmB (Fungizone, E. R. Squibb and Sons Inc., New York) powder was reconstituted with distilled water and further diluted in sterile non-pyogenic saline (0·9% w/v). For *in vitro* studies AmB (Flow Laboratories) was diluted in RPMI-1640 medium to the required concentration. The diluent, sodium deoxycholate (DOC) was prepared in a similar manner.

Mitogens. The mitogens used in the study were phytohaemagglutinin (PHA, Wellcome) and lipopolysaccharide W from *E. coli* 055:B5 (LPS, Difco). These were made up to the required concentration in RPMI-1640 medium.

Delayed-type hypersensitivity (DTH). This was essentially the method of Liew (1977). Mice were immunized by injecting 1×10^8 sheep red blood cells (SRBC) subcutaneously in the back. On the fifth day after immunization the mice were chal-

Correspondence: Dr Y. H. Thong, Department of Paediatrics, The Adelaide Children's Hospital, North Adelaide, 5006, South Australia, Australia.

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lenged with 1×10^8 sheep red blood cells (SRBC) subcutaneously in the hind footpad. Increases in footpad thickness were measured 24 hr after challenge with a dial caliper (Mercer). The degree of DTH was expressed as the percentage increase in footpad thickness.

Anti-SRBC antibody response. Mice were immunized by injecting 1×10^9 SRBC intravenously via the tail vein. All mice were bled on the fifth day after immunization and sera prepared. The anti-SRBC antibody titre was determined as follows: two-fold dilutions (0·025 ml) of the mouse sera were made in microtitre plates with saline. To each well was added 0·025 ml of 1% (v/v) SRBC. The plates were incubated at 37°C for 1 hr and then observed for haemagglutination.

Preparation of lymphocytes. Mice were killed by cervical dislocation and the spleens removed aseptically. These were gently pressed through a sieve into a petri dish containing ice-cold medium. The cell suspension was gently pipetted up and down several times, and then allowed to stand for a few minutes to allow the larger clumps of cells to settle out. The cell suspension was then carefully removed and centrifuged at 45 ***g*** for 30 sec. The supernatant containing predominantly single cells was removed and centrifuged at 1000 ***g*** for 5 min. The pelleted cells were resuspended in 0·87% ammonium chloride solution and incubated at 37°C for 5 min to lyse the erythrocytes. The spleen cells were then washed four times and resuspended in RPMI-1640 medium. Viability of the resulting cell preparation was >95%, as judged by trypan blue dye exclusion as follows: to 0·1 ml of cell suspension (2×10^6/ml) was added 0·1 ml of a 0·2% solution of trypan blue in RPMI-1640 medium, the mixture was incubated for 5 min at 37°C and the number of stained cells determined by counting in a haemocytometer.

Lymphocyte culture. Cell cultures were set up in triplicate in wells of a microtitre plate as described previously (Thong & Ferrante, 1979). Each well received 1×10^6 spleen cells in 0·2 ml of RPMI-1640 medium and either PHA or LPS at final concentrations of 0·5 μg/ml and 250 μg/ml, respectively. To the test wells was added AmB at the required concentration. Control wells received the appropriate concentrations of the diluent, DOC. The cultures were incubated at 37°C in a 5% CO_2 in air atmosphere and high humidity. All cultures were pulsed with 1 μCi of ^{3}H-TdR 6 hr before harvesting. The cells were harvested after 48 hr of incubation using a Skatron cell harvester and the samples were counted in a Packard Tricarb Scintillation Spectrometer.

RESULTS

The effect of AmB on the DTH response

Since the DTH response is a measure of cell-mediated immunity (McGregor & Kostiala, 1976), it was used in the following experiments to determine the effect of AmB on the cell-mediated immune response. Animals were divided into six groups of six. Those in the first three groups received either 2·5, 5·0 or 7·5 mg/kg of body weight of AmB daily, while the other three groups were injected with the respective amounts of DOC. Preliminary studies in our laboratory had established that DOC, at the highest concentration used in these experiments (6 mg/kg), did not affect the immunological response. Treatment was started 1 day before immunization and terminated on the day of challenge (day 5). These dosage schedules were well tolerated.

The results presented in Fig. 1 show that AmB markedly suppressed the DTH response. Significant suppression occurred in animals treated with 5·0 and 7·5 mg/kg of body weight ($0{\cdot}02 < P < 0{\cdot}05$ and $P < 0{\cdot}001$, respectively).

In order to determine whether AmB affected the priming or the manifestation phase of the DTH response, further experiments were set up to find out if AmB was acting at one or both of these phases.

The effect of AmB on the priming and manifestation phase of the DTH response

In the following experiments, the dose of AmB chosen to be injected into mice was 7·5 mg/kg of body weight because this dose gave the best suppression in the experiments described above. Mice were divided into ten groups of five. The first five groups were treated with AmB as follows: mice in groups (1), (2) and (3) were given daily treatment starting 1 day before, the same day and 1 day after priming with SRBC, respectively. Treatment was stopped on day 5. The remaining two groups were given single injections of AmB. Mice in group (4) received AmB on the same day as priming while those in group (5) were given AmB on the day of challenge (day 5).

The data presented in Fig. 2 demonstrate that AmB given after the day of priming could still inhibit the response. In fact, a single dose administered on the same day as challenge markedly inhibited the DTH response. However, a single injection given on the day of priming had no significant effect. These results indicate that AmB affected the manifestation phase of the DTH response.

The effect of AmB on the anti-SRBC antibody response

The ability of AmB to inhibit the haemagglutination antibody response to SRBC was tested in mice

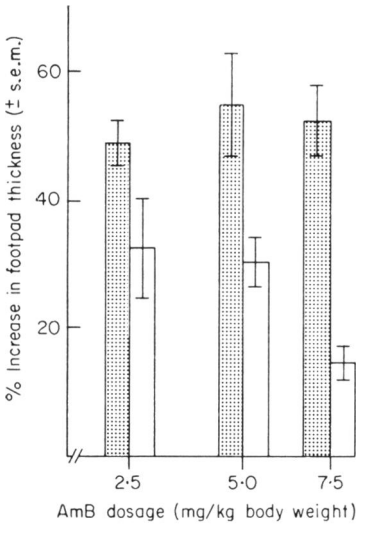

FIG. 1.

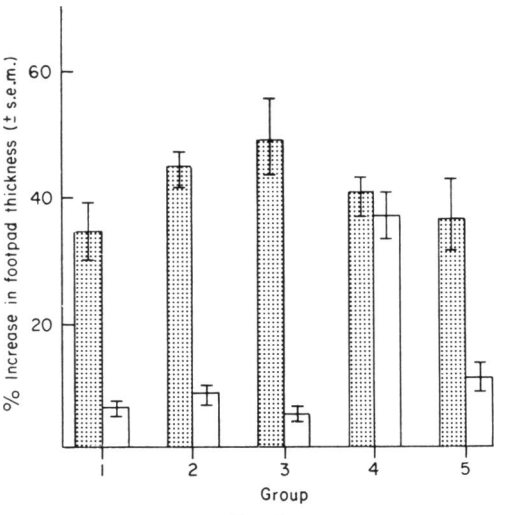

FIG. 2.

FIG. 1. The effect of daily AmB treatment on the DTH response. (▦) DOC-treated; (□) AmB-treated. Suppression was significant at 5·0 and 7·5 mg/kg of body weight ($0{\cdot}02 < P < 0{\cdot}05$ and $P < 0{\cdot}001$, respectively).

FIG. 2. The effect of AmB on the priming and manifestation phase of the DTH response. (▦) DOC-treated; (□) AmB-treated. Mice in groups (1), (2) and (3) were given daily treatment of either DOC or AmB starting 1 day before, on the same day and 1 day after priming, respectively. Those in group (4) were given a single injection on the day of priming while those in group (5) received a single injection on the day of challenge for DTH response. Significant suppression occurred in groups (1), (2), (3) and (5) which had been treated with AmB ($P < 0{\cdot}001$).

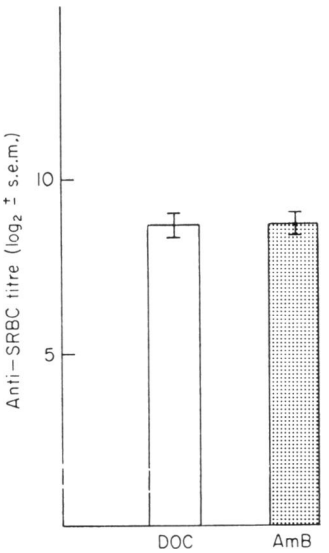

FIG. 3. The effect of AmB on the haemagglutinating anti-SRBC response in mice. Mice were treated with 7·5 mg/kg of body weight of AmB daily, beginning 1 day before priming with SRBC and terminating on day 5. Controls received the appropriate concentration of the diluent, DOC.

TABLE 1. Effect of AmB on lymphocyte proliferation induced by mitogens

AmB concentration (μg/ml)	Percentage inhibition of ^{3}H-TdR incorporation	
	PHA	LPS
0	0	0
1·0	42·4 ± 10·9*	21·5 ± 12·1†
5·0	78·8 ± 5·5‡	48·8 ± 10·0§
25·0	99·3 ± 0·2‡	96·3 ± 0·9‡

Results are expressed as mean ± s.e.m. of seven experiments.

* $0{\cdot}01 < P < 0{\cdot}02$.
† Not significant.
‡ $P < 0{\cdot}001$.
§ $0{\cdot}001 < P < 0{\cdot}01$.

treated with 7·5 mg/kg of AmB daily, beginning 1 day before priming with SRBC and terminating on day 5. Control mice were treated with the respective amounts of DOC. The results show that AmB had no effect on the antibody response to SRBC as determined by the level of circulating haemagglutinating antibody (Fig. 3).

The effect of AmB on mitogen-induced lymphocyte proliferation

In order to define more clearly the effects of AmB on the immune response, the effect of this drug on the mitogen-induced lymphocyte response was studied.

Mouse splenic lymphocytes were incubated in the presence of either PHA or LPS and varying concentrations of AmB. Control cultures were incubated with the respective quantities of DOC as that present in the AmB solution. Preliminary studies in our laboratory showed that DOC, at the highest concentration used in these experiments (20 μg/ml) did not influence lymphocyte transformation. The results showed that AmB markedly inhibited the lymphocyte response to these mitogens (Table 1). Suppression was evident at a 5 μg/ml concentration of AmB. At this concentration it had a more profound effect on the PHA than the LPS response.

The effect of AmB on lymphocyte viability

Experiments were set up to determine whether the immunosuppressive properties of AmB were related to its toxicity for lymphocytes. Splenic lymphocytes were incubated in microtitre plates in the presence of mitogens, and AmB or DOC. After 48 hr of incubation the cells were tested for viability using the

TABLE 2. Effect of AmB on lymphocyte proliferation in the presence of a suboptimal mitogen concentration

PHA concentration (μg/ml)	^{3}H-TdR incorporated (cpm)		LPS concentration (μg/ml)	^{3}H-TdR incorporated (cpm)	
	DOC	AMB		DOC	AMB
0·5	49,701 ± 2821	5923 ± 448	250·0	9843 ± 756	4825 ± 595
0·05	3488 ± 282	1519 ± 39	25·0	4775 ± 272	2216 ± 577
0·005	4248 ± 809	1785 ± 255	2·5	3142 ± 74	1604 ± 308
0·0005	2586 ± 111	1675 ± 253	0·25	2570 ± 266	1747 ± 425

Results are expressed as mean ± s.e.m. of triplicate samples.

TABLE 3. Reversibility of AmB-induced inhibition

Treatment	PHA			LPS		
	^{3}H-TdR incorporated (cpm)		Percentage inhibition	^{3}H-TdR incorporated (cpm)		Percentage inhibition
	DOC	AMB		DOC	AMB	
Non-washed	30,721±2263	5457±583	82·2*	9874±481	5013±818	49·2‡
Washed	28,395±870	14,041±1120	50·6†	11,104±721	12,191±1195	0

Results are expressed as mean± s.e.m. of triplicate samples.
Similar results were obtained in one other experiment.

* $P< 0·001$.
† $0·001< P< 0·01$.
‡ $0·01< P< 0·02$.

trypan blue dye exclusion test (see the Materials and Methods section). The results obtained demonstrated that AmB was not toxic for lymphocytes at 5 μg/ml, although some reservation should be made as to the accuracy of this test as an indicator of cell viability (Bhuyan *et al.*, 1976).

The effect of AmB on lymphocyte proliferation in the presence of suboptimal concentrations of mitogens

It has previously been reported that some drugs may demonstrate dual effects (enhancement and suppression), depending upon the dose of mitogen used (Gery & Eidinger, 1977).

Lymphocyte cultures were set up with varying concentrations of PHA or LPS. AmB was added to one set of cultures (5 μg/ml), while the control set received DOC. The results presented in Table 2 show that AmB also suppressed the lymphocyte response in the presence of suboptimal mitogen concentrations.

Reversibility of AmB inhibitory effect on lymphocytes

AmB once bound to sterol components of the membrane may cause irreversible changes to the membrane. In the following experiments lymphocytes were cultured in the presence of AmB (5 μg/ml) in culture tubes for 1 hr at 37°C. They were washed three times with medium and then added to wells in

TABLE 4. Effect of the delayed addition of AmB on mitogen-induced responsiveness

Time (hr)	Percentage inhibition of ^{3}H-TDR incorporation	
	PHA	LPS
0	80·3±6·4*	36·0±7·5†
4	67·3±6·3*	35·4±9·7‡
24	59·4±4·0*	23·4±10·4§
42	25·9±22·1§	11·4±8·7§

Results are expressed as mean± s.e.m. of triplicate samples. Similar results were obtained in one other experiment.

* $P< 0·001$.
† $0·01< P< 0·02$.
‡ $0·02< P< 0·05$
§ Not significant.

microtitre plates. DOC was added to control tubes in place of AmB. Control cell cultures in which the drug or diluent was not removed were also included. PHA or LPS was then added to the cultures.

The data in Table 3 show that the inhibitory effect of AmB on the PHA-induced response could not be reversed by washing the treated cells. In contrast, the inhibitory effect of the drug on the LPS-induced response was totally removed by washing the treated lymphocytes.

The effect of delayed addition of AmB on lymphocyte responsiveness

The inhibitory effects of AmB could be related to its action at either the initiation phase or the proliferative phase of the mitogen-induced response. Experiments were set up to determine whether AmB displayed inhibitory activity after the lymphocyte response had been initiated.

Lymphocyte cultures were set up in the presence of PHA or LPS. At different intervals of time, namely 0, 4, 24 and 42 hr, AmB (5 μg/ml) was added to the respective wells. To control cultures was added diluent (DOC) only.

The data presented in Table 4 demonstrate that AmB had a pronounced effect on the PHA- and LPS-induced response even when added 24 hr after the initiation of cultures.

DISCUSSION

Drugs that modify immunological reactivity can enhance or suppress such responses depending on the dosage schedule and route or administration, a good example being cyclophosphamide (Gill & Liew, 1978). The data presented here show that AmB, when administered on a daily schedule for 6 days, caused a marked inhibitory effect on the cell-mediated immune response (CMI), as determined by its ability to reduce the DTH response to SRBC in mice. In contrast, Blanke *et al.* (1977) reported an enhancement of DNFB responses when using a single dose of AmB at the day of priming. Further experiments carried out in the present investigation indicated that AmB acts on the manifestation phase of the DTH response. Thus, a single injection of AmB given on the day of challenge markedly suppressed the response while that given on the day of priming had no effect.

The inhibition of the expression of DTH by AmB may be related to its effect on the infiltrating cell population (T-immunoblasts and monocytes), perhaps by interacting with the cell membrane and inhibiting the movement of cells. In fact, AmB has been shown to inhibit neutrophil chemotaxis *in vitro* (Björksten, Ray & Quie, 1976; Thong & Ness, 1977).

The humoral antibody response, as measured by the levels of circulating anti-SRBC agglutinating antibody following immunization with SRBC, was not affected when mice were subjected to the same daily treatment of AmB as that which caused an inhibition of CMI, suggesting that AmB-induced immunosuppression is selective.

The interaction between AmB and the components of the immune system was studied *in vitro*, using the mitogen-induced lymphocyte blastogenic assay which is generally used as a measure of immunocompetence. The results demonstrated that AmB inhibits both the PHA- and LPS-induced murine lymphocyte response. However, the data showed that the lymphocyte response to PHA was much more sensitive to AmB than was the LPS-induced lymphocyte response. The inhibitory effect of AmB on the PHA-induced response was irreversible while that on the LPS-induced response could be reversed by washing the treated lymphocytes. From these results, it would seem that AmB has a greater effect on the T cell than the B cell response. This may explain the *in vivo* selective effect of the drug.

The data presented show that AmB does not act by preventing the binding of PHA and LPS to the surface membrane of the lymphocyte, since marked inhibition could be demonstrated when the drug was added 24 hr after the addition of mitogens to cultures. Its effect would seem, therefore, to be on blast cells.

The results of the present studies clearly demonstrate that AmB, given in a multiple dosage schedule, markedly suppresses CMI. Since CMI is an important defence mechanism against fungal infections (Gentry & Remington, 1971; Cutler, 1976; Rogers, Balish & Manning, 1976; Tewari *et al.*, 1977; Howard & Otto, 1977; Graybill & Taylor, 1978), consideration must be given to the immunosuppressive

properties of this drug during chemotherapy of systemic fungal infections. These infections usually occur in patients with cancer or other debilitating illnesses, and further immunosuppression by AmB may militate against recovery from infection.

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