Immunology 1982 **47** 115

Inhibition of proliferation of a murine myeloma cell line and mitogen-stimulated B lymphocytes by the antibiotic amphotericin B (Fungizone)

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Accepted for publication 24 February 1982

Summary. Amphotericin B at the concentration normally used for routine suppression of fungal infection in tissue culture strongly inhibits the proliferation of NS1/1 myeloma cells and the LPS-induced activation of B lymphocytes from mouse spleen. The proliferation of T lymphocytes induced by concanavalin A (Con A) was less affected by the antibiotic, indicating that B-lymphocyte proliferation was preferentially inhibited. The unexpected sensitivity of B-lymphoid cells to amphotericin B precludes its use as an anti-fungal agent in the production of hybridomas from fusions between these cells.

INTRODUCTION

Amphotericin B (AmB), marketed as Fungizone, is an anti-fungal polyene antibiotic produced by several species of *Streptomyces* (Little, Blanke, Valeriote & Medoff, 1978). It has been used therapeutically, although not without side effects (Boxer, Ingraham, Allen, Oseas, & Baehner, 1981), and is widely used in tissue culture to combat infections of yeasts and moulds. AmB is thought to act by binding to membrane sterols. Its selective toxicity for fungi can be accounted for by the interaction with ergosterol, present in fungal membranes, being stronger than that with cholesterol, the main sterol in animal cell membranes. The antimycotic agent affects cell permeability towards a variety of ions and molecules including other drugs. Its general properties and specific immunoadjuvant and anti-tumour activities have been reviewed by Little *et al.* (1978). Other examples of AmB's immunomodulating properties are described by Stewart, Spagnuolo & Ellner (1981); Olds, Stewart & Ellner (1981); and Shirley & Little (1979a, 1979b).

To prevent loss of potentially valuable antibody-secreting hybridomas following polyethylene glycol-induced fusions between murine myeloma cells and immune spleen lymphocytes, we included AmB in the selective hypoxanthine-aminopterin-thymidine (HAT) medium used. However, when fusion mixtures were plated into HAT medium containing 2·5 μg/ml AmB, no hybrid colonies developed, When half of a fusion mixture was put into HAT medium with AmB and the rest into medium without the antimycotic agent, hybrid colonies, some producing specific antibodies, only developed in the absence of AmB. This observation led to the studies described in this paper

Abbreviations: AmB, amphotericin B; Con A, concanavalin A; HAT, hypoxanthine-aminopterin-thymidine; HEPES, 4 [2- hydroxyethyl]-1-piperazine-ethanesulphonic acid; FCS, foetal calf serum; LPS, lipopolysaccharide; ID_{50}, dose giving 50% inhibition.

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0019-2805/82/0900-0115$02.00

which demonstrate that both the mouse myeloma cell line and splenic B lymphocytes were highly sensitive to this antibiotic.

MATERIALS AND METHODS

Materials

RPMI 1640 (ten times) medium and Hanks's balanced salt solution (ten times) were obtained from Gibco Europe, Paisley, Scotland, and L-glutamine, penicillin plus streptomycin, non-essential amino acids and heat-inactivated foetal calf serum (FCS) from Flow Labs, Irvine, Scotland. [Methyl-^{3}H]-thymidine (specific activity 5 Ci/mmol) was obtained from Amersham International, Amersham, as were L-[U-^{14}C]-leucine (specific activity 339 mCi/mmol) and L-^{35}S]-methionine (specific activity 1000 Ci/mmol). HEPES (4[2-hyroxyethyl]-1-piperazine-ethane sulphonic acid) was purchased from BCL, Lewes.

Amphotericin B (or Fungizone, registered trade mark of E.R. Squibb & Sons) was supplied by Gibco Europe as a lyophilysed powder prepared in water. It was rehydrated to 250 μg/ml with sterile double-distilled water and stored at $-20°$. This stock was used at 1/100 dilution (2·5 μg/ml) in complete culture medium or was diluted to ten times the required concentration with serum-free medium.

Concanavalin A (Con A) obtained in crystalline form from Miles-Yeda, Rehovoth, Israel, was dissolved in serum-free medium and filtered before use. Lipopolysaccharide (LPS; Bacto LPS.W. *E. coli*, 0127:B8) was from Difco Laboratories West Molesey. The lyophilysate was rehydrated to 10 mg/ml with sterile 0·15 M NaCl in double-distilled water.

NS1/1 Myeloma cultures

The NS1/1 derivative of the P3.X63.Ag8 myeloma, of BALB/c origin, was maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol, 50 i.u./ml penicillin and 50 μg/ml streptomycin (RPMI supp.) plus 10% FCS. The cells were grown in Nunc tissue culture flasks at densities between 5×10^4/ml and 5×10^5/ml with a humid 5% CO_2 in air atmosphere of 37°.

Fusion of NS1/1 myeloma cells with immunized BALB/c spleen lymphocytes

Polyethylene glycol-mediated fusions of spleen cell preparations from immunised BALB/c mice with NS1/1 myeloma cells were carried out according to the procedure of Lemke, Hämmerling, Höhmann & Rajewsky (1978), with minor modifications. The fusion mixture was dispensed dropwise into Costar 24 well flat-bottomed plates containing 2 ml of selective medium per well. This was RPMI (supp.) plus 20% FCS with Littlefield's concentrations of hypoxanthine, aminopterin and thymidine (HAT medium; Littlefield 1964). AmB, if included in the HAT medium, was present at 2·5 μg/ml. The appearance of colonies was assessed 10–20 days post fusion.

Growth curve for NS1/1 cells

Suspensions of myeloma cells at 5×10^4/ml were prepared in RPMI (supp.) plus 10% FCS and the concentrations of AmB indicated. One millilitre aliquots of the cell suspensions were dispensed to Nunc 4 well dishes and the plates were incubated for 1–7 days. At approximately 24 hr intervals, the cultures were observed using an inverted microscope and after resuspension by pipetting, the cells were counted. For each AmB concentration cells from duplicate 1 ml cultures were counted, except when they had passed the confluence stage, which occurred at a density of about 10^6 cells/ml.

Cloning of NS1/1 myeloma

This was carried out in Nunc 96 well flat-bottomed microtitre plates using RPMI (supp.) plus 15% FCS. For each dose of AmB, three concentrations of NS1/1 cells were used, averaging 5, 2·5 and 1·25 cells per 200 μl well. Serial two-fold dilutions of the cells were made in serum-free medium. These were then further diluted with medium, AmB and FCS to give the cell, AmB and serum concentrations stated. For each AmB and NS1/1 concentration, 200 μl aliquots of cell suspension were dispensed to 24 wells of a 96-well plate. The plates were incubated for at least a week and the numbers of wells containing macroscopically or microscopically visible clones determined. Cloning efficiency and relative survival values were calculated from the numbers of positive and negative wells at each cell plating density and AmB concentration according to the method of Kraemer, Waters & Buchanan (1980).

Preparation and culture of mouse spleen cells

Male Porton strain mice, 3 months old, were killed by cervical dislocation and the spleens removed aseptically. Unseparated preparations of the spleen cells were made in Hanks's balanced salt solution containing 1 mM HEPES at room temperature.

Spleen cells were cultured at 10^6/ml in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 5×10^{-2}M mercaptoethanol, non-essential aminoacids, 50 i.u./ml penicillin and 50 μg/ml streptomycin plus 5% FCS.

Triplicate 1 ml cultures of spleen cells in the presence or absence of AmB and mitogens were prepared in 65×15 mm glass tubes with oxoid caps. Incubation was at 37° in a humid atmosphere of 5% CO_2 in air. Protein synthesis was assayed by incorporation of radiolabelled amino acids, either [^{35}S]-methionine or [^{14}C]-leucine, into acid-insoluble material after 24 hr of culture and DNA synthesis by incorporation of [^{3}H]-thymidine into acid-insoluble material at 48 hr of culture. One microcurie of radiolabelled precursor in 100 μl of serum-free medium was added to each culture 4 hr before termination. Incubation was stopped by adding 2 ml of cold 0·15 M NaCl to each tube. After washing in cold 0·15 M NaCl, 3 ml of cold, 10% (w/v) trichloracetic acid was added to the cell pellets and the precipitates filtered onto glass-fibre discs. These were dried and counted in toluene based scintillant using a Beckman model LS 233 liquid scintillation counter.

RESULTS

Inhibition of growth of NS1/l myeloma cells by amphotericin B

Direct assessment of the effects of different concentrations of AmB on the proliferation of NS1/l cells at 24 hr intervals after AmB addition showed that concentrations up to 1 μg/ml did not seriously affect cell replication (Fig. 1). Under the culture conditions used the cells formed a confluent layer on the bottoms of the wells at a density of 10^6/ml, but continued to divide. This stage was reached after 72–96 hr of culture in the presence of up to 1 μg/ml AmB. However, proliferation of the NS1/l cells was substantially inhibited by 2·5 μg/ml AmB. Cell replication was inhibited by at least 75% during the first 72 hr of culture. Proliferation then resumed at approximately the normal rate. This may have been due either to the multiplication of AmB-resistant variants within the NS1/l cell population or reduction of the effective AmB concentration by decomposition and/or inactivation (see Gibco Europe Product Catalogue 1981–82, p. 35). When the concentration of AmB was raised to 5 μg/ml the cell density initially fell below the seeding concentration, but some proliferation occurred after 6 days in culture. Addition of 10 μg/ml AmB apparently killed most of the myeloma cells. Microscopic examination of the cultures revealed much cell debris with only very few intact cells and no evidence of proliferation was seen within the duration of the experiment.

Cloning by limiting dilution of the NS1/l myeloma cells in the presence of different concentrations of AmB was carried out as an indirect assay of the antimycotic's deleterious action on the cells. Cloning efficiencies, i.e. the ability of cells to initiate microcultures, and the relative values in the presence of AmB were calculated according to the method of Kraemer *et al.* (1980) (Fig. 2). The mean cloning efficiency of NS1/l myeloma cells in the absence of AmB, determined at initial plating densities averaging 5, 2·5 and 1·25 cells per well, was 31%. Addition of AmB at up to 1 μg/ml did not reduce this—in fact a slight enhancement was observed in the experiment shown—but the relative survival decreased rapidly at higher concentrations. The dose giving 50% inhibition (ID_{50}) for cloning of NS1/l myeloma cells was 2·15 μg/ml AmB, while at 5 μg/ml the relative survival value had fallen below 5%. No clones were formed at 10 μg/ml AmB.

Effects of amphotericin B on the mitogenic responses of mouse spleen lymphocytes

To determine whether the high degree of sensitivity of NS1/l cells to AmB was restricted to this cell line or a more general property of B lymphocytes, the effects of AmB on the response of murine lymphocytes to the B-lymphocyte mitogen LPS were also studied. In addition to its effects on the LPS-induced stimulation of DNA synthesis after 48 hr (Fig. 3a) modifications by AmB of the earlier stimulation of protein synthesis were also determined (Fig. 3b). At all LPS concentrations studied, 2·5 μg/ml AmB strongly inhibited the mitogen-induced stimulation of DNA synthesis. The extent of this inhibition was comparable with that of NS1/l myeloma cell proliferation noted above in the presence of the same AmB concentration. The time course of the response was not affected, maximal [^{3}H]-thymidine incorporation being observed 48–72 hr after stimulation in the presence or absence of AmB (results not shown). There was some variation from animal to animal in both the degree of mitogenic stimulation by LPS and its inhibition by AmB, but strong inhibition was always observed. Optimum stimulation was seen over quite a wide range of LPS concentrations, and there was no evidence that the degree of inhibition by AmB was influenced by the mitogen concentration used (Fig. 3a). The earlier

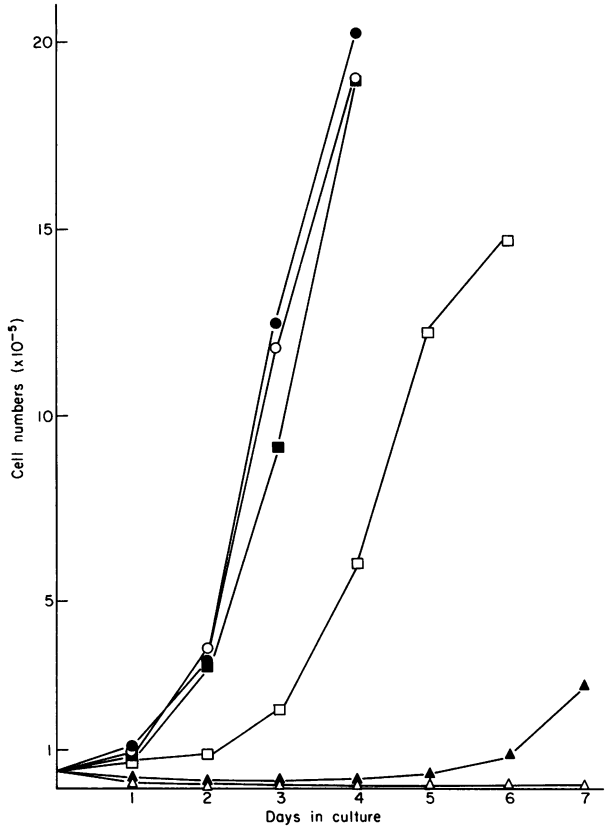

Figure 1. Growth of NS1/1 myeloma cells in the presence of AmB. Replicate 1 ml cultures containing different concentrations of AmB and initially 5×10^4 NS1/1 cells were set up as described in Materials and Methods. At approximately 24 hr intervals the numbers of cells present in duplicate cultures for each AmB dose were determined. Concentrations of AmB (μg/ml): (●) 0; (○) 0·25; (■) 1·0; (□) 2·5; (▲) 5·0; (△) 10·0. In Figs 1–5 standard error bars have been omitted from diagrams, but are mostly contained within the symbols used.

stimulation of [^{35}S]-methionine incorporation into protein by LPS was also significantly inhibited by 2·5 μg/ml AmB, but the magnitude of the inhibition was lower than the later effect on DNA synthesis (Fig. 3b). The degree of inhibition by AmB did not vary greatly with the LPS concentrations used or if protein synthesis was assessed by measuring the incorporation of [^{14}C]-leucine instead of [^{35}S]-methionine.

When similar experiments were carried out to determine the effects of AmB on the response of murine lymphocytes to the T-cell mitogen Con A, no significant inhibition of the mitogen-dependent increase in DNA synthesis was observed (Fig. 4a), although 60% inhibition was seen when the AmB concentration was raised to 10 μg/ml and 75% inhibition at 25 μg/ml. Similar results were obtained when [^{35}S]-methionine incorporation into protein was determined 24 hr after Con A addition (Fig. 4b).

The effects of a range of AmB concentrations on DNA and protein synthesis by mouse spleen lymphocytes incubated with or without 20 μg/ml LPS are shown in Fig. 5. This concentration of LPS is at the lower end of the optimum dose range so that any enhancement of lymphocyte activation due to AmB could be seen. DNA synthesis induced by LPS was more sensitive to AmB than the proliferation of NS1/1 cells as substantial inhibition was seen with drug concentrations as low as 0·25 μg/ml (41% inhibition, see Fig. 5a). The low level of DNA synthesis seen in cultures without mitogen was also inhibited by AmB.

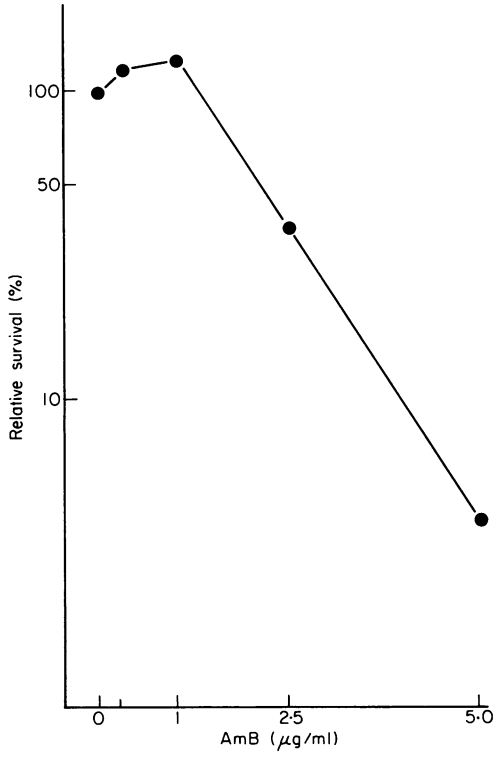

Figure 2. Effect of AmB on cloning efficiency of NS1/1 myeloma cells. Cloning by limiting dilution of NS1/1 cells in the presence of AmB was carried out as described in Materials and Methods. Microculture initiation was assessed 9 days after seeding and calculations made using the method of Kraemer *et al.* (1980).

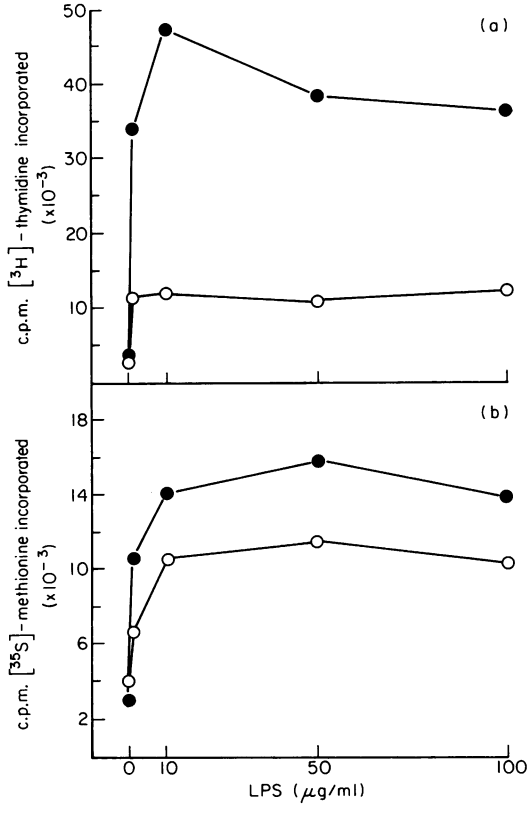

Figure 3. Effect of 2·5 μg/ml AmB on LPS stimulation of mouse spleen B lymphocytes. (a) Incorporation of [^{3}H]-thymidine into DNA was assessed from 44 to 48 hr and (b) [^{35}S]-methionine incorporation into protein from 20 to 24 hr. Triplicate cultures were set up as in Materials and Methods, (●) without AmB and (○) with 2·5 μg/ml AmB.

No stimulation comparable with that reported by Hammarström & Smith (1976) was observed at any concentration tested. The effects of AmB on [^{35}S]-methionine incorporation into protein were again smaller, but qualitatively similar (Fig. 5b). The drug did not affect the rate of protein synthesis by unstimulated cultures significantly at concentrations up to 2·5 μg/ml and again only inhibitory effects were seen at higher concentrations (maximum of 40%). No stimulatory effects of 10 μg/ml AmB, either alone or in combination with Con A or LPS, were observed in any of ten experiments.

DISCUSSION

Production of hybrid colonies after fusion of the NS1/1 derivative of a mouse myeloma P3.X63.Ag8 with immune mouse spleen cells was prevented by 2·5 μg/ml AmB present in the HAT medium. This could be explained by AmB inhibiting proliferation of either or both of the fusion partners; specific inhibition of hybridoma replication or due to toxicity of the antimycotic agent.

The NS1/1 variant of the P3.X63.Ag8 mouse myeloma cell line is very sensitive to AmB, as could be seen from the growth curve and cloning experiments, with an ID_{50} of less than 2·5 μg/ml AmB. This contrasts with a variety of other cell lines of mouse and human origin, grown both in suspension and as monolayers in different types of medium, which have ID_{50}s of 25 μg/ml AmB or more (Gibco Europe Product Catalogue 1981–82, p. 36).

To study the effects of AmB on proliferation of

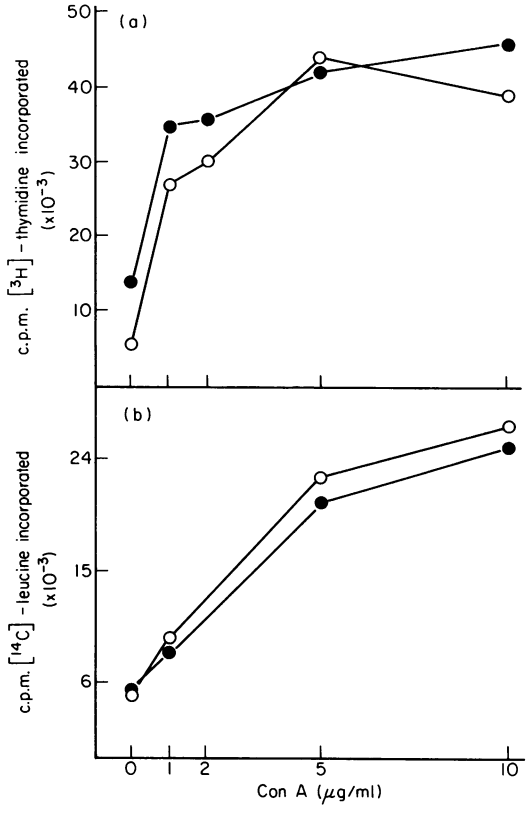

Figure 4. Effect of 2·5 μg/ml AmB on Con A stimulation of mouse spleen T lymphocytes. (a) incorporation of [^{3}H]-thymidine into DNA was assessed from 44 to 48 hr and (b) [^{14}C]-leucine incorporation into protein from 20 to 24 hr. Triplicate cultures were set up as in Materials and Methods, (●) without AmB and (○) with 2·5 μg/ml AmB.

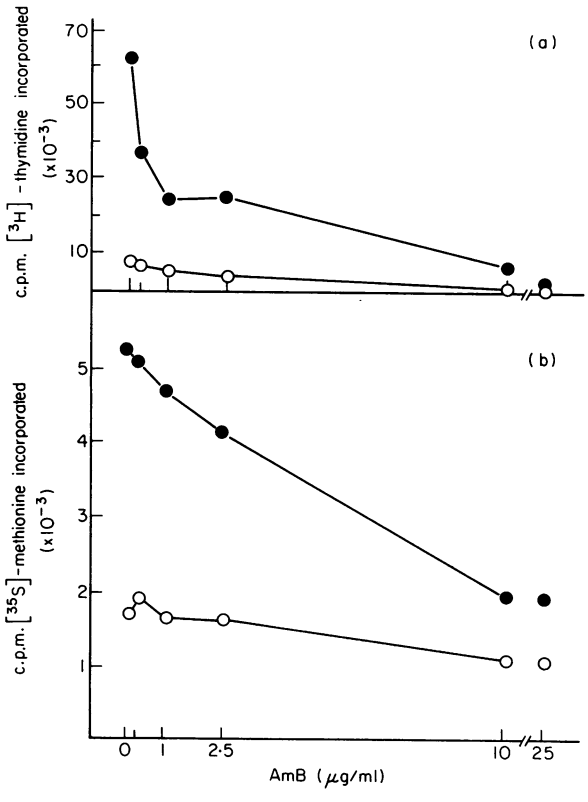

Figure 5. Effects of different concentrations of AmB on stimulation of murine B lymphocytes by 20 μg/ml LPS. (a) Incorporation of [^{3}H]-thymidine into DNA was assessed from 44 to 48 hr and (b) [^{35}S]-methionine incorporation into protein from 20 to 24 hr. Triplicate cultures were set up as in Materials and Methods, (●) without LPS, (○) with 20 μg/ml LPS.

mouse spleen lymphocytes the population specific mitogens, LPS(B) and Con A(T), were used thereby enabling unseparated spleen cell preparations (as were used in the polyethylene glycol-induced fusion experiments) to be studied. DNA synthesis by activated B lymphocytes was markedly inhibited by less than 2·5 μg/ml AmB. At 2·5 μg/ml of AmB DNA synthesis by the stimulated B cells was reduced by 75% of control values, but the same dose of antimycotic did not seriously affect the same process in activated T lymphocytes or unstimulated spleen cells. Macromolecule synthesis by the latter spleen cell populations was reduced by higher doses of AmB.

These results suggest that the marked sensitivity of the NS1/1 myeloma to AmB is not confined to this cell line but is a general property of murine B lymphocytes.

Inhibition of proliferation of NS1/1 myeloma cells and mitogeneitically stimulated mouse spleen lymphocytes by AmB could account for the absence of colonies if it is present in the HAT medium. We have not yet studied growth of NS1/1 based hybridomas in the presence of different concentrations of AmB. However, hybridoma cells are generally more sensitive to alterations in culture conditions than their parent myelomas, antibody secreting hybrids tending to lose chromosomes and their ability to produce specific monoclonal antibodies.

The dose of AmB used in cultures cannot be reduced much below 2·0 μg/ml without decreasing its efficiency as an antimycotic; minimum inhibitory concentrations for several genera of yeasts are around 1·9 μg/ml (Gibco Europe Product Catalogue 1981–82, p35).

It might be possible to produce a variant of the NS1/1 line which is more resistant to AmB than the parent myeloma, but is still a suitable fusion partner, i.e. will give rise to a high proportion of stable antibody producing hybrids. Abo & Balch (1981) who grew their mouse myeloma cell line (P3.X63.Ag8.653) in the presence of 2·0 μg/ml AmB were able to obtain a stable hybridoma secreting a specific monoclonal antibody from a fusion carried out in the presence of AmB. Routine inclusion of AmB in such cultures would reduce the loss of potentially valuable hybridomas due to fungal contamination.

The antimycotic agent affected protein synthesis less than DNA synthesis. This may be because there is differential inhibition of metabolic processes by AmB and/or that such effects are time dependent, events occurring soon after activation being affected less than later ones. AmB at 2·0 μg/ml has been shown to activate monocytes and suppressor T cells in human peripheral blood mononuclear cell preparations, thereby inhibiting responses to the T-cell mitogen PHA (Stewart *et al.*, 1981). Such activation of macrophages and suppressor T lymphocytes (by AmB) in a mixed spleen-cell population could contribute to reduced mitogenic stimulation of subclasses of cells within that population. However, the antimycotic's inhibition of proliferation by the NS1/1 myeloma cell line indicates that it can act directly on sensitive cells. This probably involves interaction(s) of AmB with components of the plasma membrane triggering off a variety of reactions within the cells. To elucidate the nature of this polyene antimycotic's interactions with plasma membranes of different subclasses of lymphoid cells and those of the monocyte-macrophage lineage, studies must be made on purified cell types as well as mixed populations.

AmB has been reported to be a B-cell mitogen for mouse spleen lymphocytes with an optimum concentration of 10 μg/ml (Hammarström & Smith, 1976). We found that over the concentration range from 0·25 to 25·0 μg/ml, AmB was not mitogenic for mouse spleen cells, either alone or in conjunction with LPS and Con A at slightly below optimum concentrations. Occasionally there was slight enhancement of incorporation of radiolabelled precursors into macromolecules at 0·25 μg/ml AmB, but this increase was never more than 20%. On the contrary, AmB usually inhibited protein and DNA synthesis by mouse spleen cells cultured with FCS alone or with mitogens. The lack of mitogenic activation of the spleen lymphocytes by AmB in these experiments was probably not due to use of the wrong concentrations of the antimycotic since doses of 0·1–10·0 μg/ml were all reported to be stimulatory by Hammarström & Smith, (1976). Also they reported that the peak response of DNA synthesis, assayed by incorporation of [^{3}H]-thymidine, occurred 48 hr after culture initiation. However, their stock AmB solution was made up in dimethyl-sulphoxide, which although not toxic at the optimum AmB concentration, may have had side effects either alone or together with the antimycotic agent.

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