ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1974, p. 529-533

Vol. 6, No. 5
Printed in U.S.A.

Effect of Amphotericin B and Clotrimazole on Lymphocyte Stimulation

ARNE TÄRNVIK AND STEFFAN ÅNSÉHN
Department of Clinical Bacteriology, the Medical School, Linköping, Sweden

Received for publication 15 July 1974

Human lymphocytes were stimulated in vitro by phytohemagglutinin, concanavalin A, pokeweed mitogen, purified protein derivative-tuberculin, and allogenic cells. The deoxyribonucleic acid synthesis of the lymphocytes was inhibited in increasing degree by 4 to 8 μg of amphotericin B per ml of culture irrespective of lymphocyte stimulant used. The effect of clotrimazole on the deoxyribonucleic acid synthesis varied between experiments with different and also between experiments with the same stimulant. Ten micrograms of clotrimazole per ml was generally inhibiting, whereas in one experiment 2 μg or more per ml inhibited the purified protein derivative-induced deoxyribonucleic acid synthesis. The effects of amphotericin B and clotrimazole were neutralized by serum.

Amphotericin B is a polyene antibiotic with an effect on fungi and other organisms containing sterols (1). Clotrimazole, bis-phenyl-2-chlorophenyl-1-imidazolyl methane, another antifungal agent, also seems to act by interference with the cell membrane (13).

Amphotericin B has a narrow therapeutic interval. In high concentrations it may cause hypokalemia and renal acidosis, possibly implicating an effect on the permeability of human cell membranes (7, 14, 17). In therapeutic concentrations amphotericin B has been reported not to affect various mammalian cell lines in vitro (12). The clinical side effects of clotrimazole as well as its effect on mammalian cells in vitro are virtually unknown.

The effect of these antimycotics on human immune mechanisms should be evaluated. An interference with immune mechanisms may be deleterious especially during infections. In the host defense against fungal infections, cell-mediated (thymus-dependent) immune reactions are believed to be more important than production of humoral antibody (20). An initial event in immune reactions is the stimulation of circulating lymphocytes, leading to proliferation and differentiation of the lymphocytes into immune effector cells. Fortunately, lymphocyte stimulation seems to be an ideal tool in cytotoxic studies. It can be used in vitro under carefully controlled conditions and it can be easily and sensitively assayed as deoxyribonucleic acid (DNA) synthesis of the lymphocytes. Lymphocyte stimulation has often been used when testing immunosuppressive agents (19).

In the present study lymphocytes were stimulated in vitro by various agents. The effect of amphotericin B and clotrimazole on the DNA synthesis of the lymphocytes was studied.

MATERIALS AND METHODS

Gelatin was a gift from Kind & Knox Gelatin Co., Camden, N.J. Ficoll was obtained from Pharmacia AB, Uppsala, Sweden, and sodium metrizoate from Nyegaard & Co., Oslo, Norway. Tissue culture medium 199 F (TCM 199) from GIBCO, Grand Island, N.Y. was diluted in *N*-2-hydroxyethyl-piperazine-*N'*-2'-ethanesulfonic acid buffer from Schwarz/Mann, Orangeburg, N.Y. according to Bach et al. (2). Phytohemagglutinin P (PHA-P) was from Difco, concanavalin A (con A) from Miles Laboratories Ltd., Stoke Poges, Buckinghamshire, England, pokeweed mitogen (PWM) from GIBCO, and purified protein derivative (PPD)-tuberculin from Statens Seruminstitut, Copenhagen, Denmark. Amphotericin B (Fungizone) was obtained from Squibb AB, Lidingö, Sweden, and clotrimazole (Bay b 5097) was a gift from Bayer-Farma AB, Stockholm, Sweden. Falcon Micro Test II tissue culture plates were from Gateway International, Los Angeles, and [*methyl*-^{3}H]thymidine (specific activity, 2.0 Ci/mmol) from The Radiochemical Centre, Amersham, England.

Lymphocyte separation. Venous blood from healthy blood donors was defibrinated by gentle agitation with glass beads. Lymphocytes were separated by gelatin sedimentation (5, 6) followed by centrifugation on a layer of Ficoll-isopaque (4). The lymphocytes were washed three times in TCM 199-2% autologous serum and suspended in TCM 199-30% autologous serum.

Lymphocyte cultures. PHA, con A, PWM, and

PPD were solubilized at 1 mg/ml in saline and kept frozen at −20 C. One of the stimulants was added to a lymphocyte suspension, 100 μliters of which was mixed with 100 μliters of a test solution in each well of a tissue culture plate. The final concentration of PHA was 20 μg/ml of culture and of the other stimulants 40 μg/ml, at which concentrations stimulation had been tested to be about optimal. The final lymphocyte density was 10^6/ml. The culture medium contained 50 U of benzylpenicillin once it had been found not to affect the results.

Mixed lymphocyte cultures (MLC) were prepared by adding the same number of lymphocytes from two individuals to a suspension, which was then used in the same way as other lymphocyte suspensions but without further addition of stimulants. The final lymphocyte density was 10^6/ml.

Amphotericin B was diluted in TCM 199 to 1.0 to 20 μg/ml. Control solutions containing sodium deoxycholate and phosphate buffer in TCM were also prepared.

Clotrimazole was solubilized at 2 mg/ml in 70% (vol/vol) ethanol and further diluted in TCM 199 to 40, 30, and 20 μg of clotrimazole per ml, giving the ethanol concentrations of 1.4, 1.05, and 0.7%, respectively. Solutions containing 16 to 1 μg of clotrimazole per ml in TCM 199-0.7% ethanol were also prepared. Control solutions contained 1.4, 1.05, 0.7, and 0% ethanol in TCM 199.

Each microplate was closed with a film and incubated at 37 C in humidified air for 3 to 4 (PHA, con A) or 6 to 7 (PPD, PWM, MLC) days. Four hours before the end of the incubation period, 0.4 μCi of [^{3}H]thymidine in 20 μliters of saline was added to each culture. Cultures were washed and precipitated onto glass fiber filters by a semi-automatic multiple-sample processor (11). The filters were placed in 10 ml of scintillation fluid [0.3 g of dimethyl-1,4-bis-(5-phenyloxazolyl)-benzene, 5 g of 2,5-diphenyloxazole to 1,000 ml of toluene] and counted in a scintillation counter.

RESULTS

The lymphocyte response to PHA, con A, PWM, PPD, and in MLC to allogenic cells was estimated in the presence of 0 to 8 or 10 μg of amphotericin B per ml of culture (Fig. 1 and 2). The incorporation of [^{3}H]thymidine into DNA decreased with increasing dose of amphotericin B similarly irrespective of stimulant used. In the presence of 4 μg/ml, the incorporation was significantly lower than in the absence of amphotericin B.

The commercially available form of amphotericin B that was used contained sodium deoxycholate as a solubilizer. The effect of various concentrations of sodium deoxycholate on the lymphocyte response to two stimulants, PHA and con A, was investigated (Table 1). In the range of 8.2 to 1.6 μg/ml, corresponding to the amphotericin B dose of 10 to 2 μg/ml, sodium deoxycholate did not affect the incorporation of [^{3}H]thymidine into DNA.

The lymphocyte response to PHA, con A, PWM, PPD, and allogenic cells was also estimated in the presence of 0 to 20 μg of clotrimazole per ml of culture (Fig. 2 and 3). Clotrimazole at 20 μg/ml inhibited the incorporation of [^{3}H]thymidine into DNA completely in experiments with all stimulants but PHA, in which the incorporation was only partly inhibited. The lowest concentration of clotrimazole inhibiting the incorporation varied not only between experiments with various stimulants but also between experiments with the same stimulant. Clotrimazole at 10 μg/ml was generally inhibitory, whereas in one experiment a concentration as low as 2 μg/ml inhibited the incorporation induced by PPD.

Serum was found to have influence upon the effect of amphotericin B as well as of clotrimazole on the lymphocyte response to PHA. In the presence of 5% serum the incorporation of

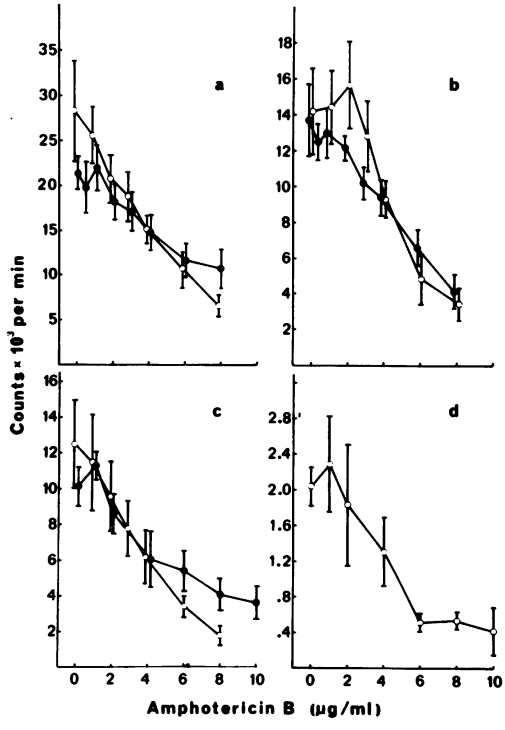

FIG. 1. *Effect of amphotericin B on the lymphocyte response to (a) PHA, (b) con A, (c) PWM, and (d) PPD. The incorporation of [^{3}H]thymidine into DNA was measured. The two lines (open and closed circles) represent experiments on lymphocytes from two donors. The means and standard deviations of five cultures are indicated.*

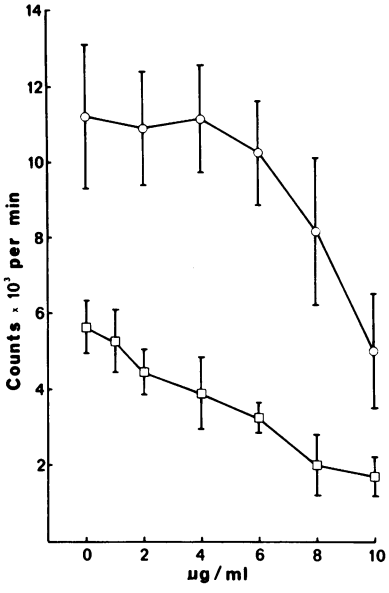

FIG. 2. *Effect of amphotericin B (□) and clotrimazole (○) on the mixed lymphocyte culture. Lymphocytes from different donors were used in the two experiments. The incorporation of [³H]thymidine into DNA was measured. The means and standard deviations of five cultures are indicated.*

TABLE 1. *Effect of amphotericin B and sodium deoxycholate on the lymphocyte response to (i) PHA and (ii) con A. The incorporation of [³H]thymidine into DNA was measured.*

Substance added		Counts × 10³/min ± standard deviation of five cultures	Substance added (sodium deoxycholate [µg/ml])	Counts × 10³/min ± standard deviation per five cultures
Amphotericin B (µg/ml)	Sodium deoxycholate (µg/ml)			
(i) 10	8.2	5.2 ± 0.6	8.2	18.7 ± 2.8
8	6.6	7.5 ± 0.7	6.6	19.0 ± 1.1
6	4.9	9.8 ± 1.2	4.9	19.3 ± 2.0
4	3.3	12.7 ± 1.1	3.3	21.7 ± 2.6
2	1.6	14.3 ± 2.5	1.6	20.0 ± 1.5
0	0	19.3 ± 2.3		
(ii) 10	8.2	0.9 ± 0.3	8.2	10.2 ± 2.2
8	6.6	2.6 ± 0.6	6.6	9.5 ± 1.1
6	4.9	3.7 ± 0.6	4.9	10.2 ± 1.5
4	3.3	6.1 ± 1.3	3.3	12.4 ± 3.2
2	1.6	6.9 ± 1.8	1.6	11.2 ± 2.5
0	0	8.7 ± 2.6		

radioactivity was more readily inhibited by both antibiotics than at 15% serum, whereas in the presence of 45% serum the incorporation was affected only slightly or not at all by the antibiotics at all concentrations tested (Fig. 4).

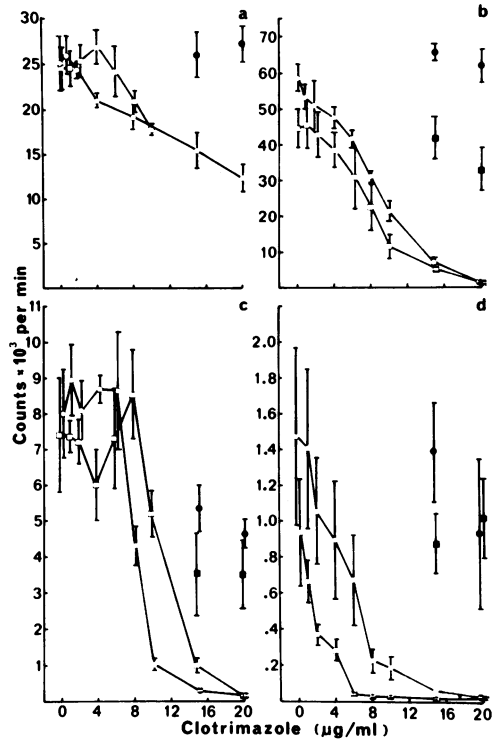

FIG. 3. *Effect of clotrimazole on the lymphocyte response to (a) PHA, (b) con A, (c) PWM, and (d) PPD. For each stimulant two experiments (circle and square) are shown representing experiments on lymphocytes from two donors. All cultures which contained 10 µg or less of clotrimazole per ml also contained 0.7% (vol/vol) ethanol. Control cultures indicated by filled symbols lacked clotrimazole but contained ethanol of the same concentration as did cultures indicated by open symbols on equal abscissa. The incorporation of [³H]thymidine into DNA was measured. The means and standard deviations of five cultures are indicated.*

DISCUSSION

PHA, con A, and PWM are plant proteins which stimulate lymphocytes irrespectively of pre-sensitization, whereas the lymphocyte response to PPD is secondary to previous contact with tubercle bacilli or BCG vaccine. PHA and con A stimulate most if not all T cells (thymus-derived lymphocytes). PWM may stimulate not only T cells but also B cells (bursa-equivalent lymphocytes). PPD and allogenic cells stimulate a smaller fraction of T cells than do the other agents. Thus the present assays measured T cell function mainly.

The present results do not indicate that in therapeutic concentrations amphotericin B inhibits lymphocyte proliferation. Common ther-

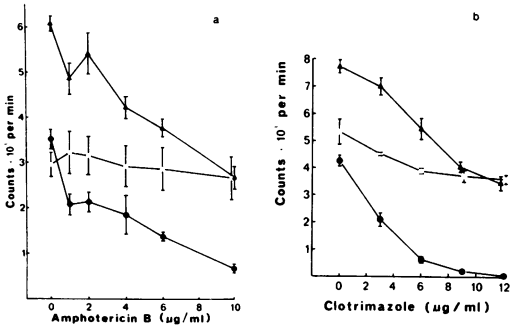

FIG. 4. *Effect of (a) amphotericin B and (b) clotrimazole on the lymphocyte response to PHA. Lymphocytes were incubated in medium containing 5 (●), 15 (▲), and 45 (○) % serum. The incorporation of [³H]thymidine into DNA was measured. The means and standard deviations of five cultures are indicated.*

apeutic doses of amphotericin B are 0.6 to 1.0 mg/kg, producing peak serum concentrations of 0.4 to 2.0 µg/ml (8). Even higher therapeutic doses or rapid intravenous infusion seem to give serum levels lower than 3 µg/ml (3, 9). Thus, in therapeutic concentrations lymphocyte stimulation was only slightly or not at all inhibited by amphotericin B.

At 4 to 10 µg/ml, amphotericin B inhibited lymphocyte stimulation. This inhibition was completely neutralized when the serum concentration of the culture medium was raised from 15 to 45%. The antifungal effect of amphotericin B is also inhibited by serum, which has been reported to be due to sterol compounds of serum (10, 15).

Due to the inhibition by serum of the effect of amphotericin B it is difficult to compare reports on the susceptibility of various cells to the drug. Anyway, available data indicate that the minimal concentrations inhibiting the growth of various yeasts range from about 0.1 to 2 µg/ml (16; S. Ånséhn, Castellania, in press). Thus, yeasts seem to be more susceptible to the drug than are lymphocytes. However, other mammalian cells such as mouse fibroblasts, HeLa cells, and Ehrlich ascites cells cultured in medium containing 10% serum have been reported resistant to as high a concentration as 25 µg/ml (18). Again, when serum was absent, the mouse fibroblasts were affected by 2.5 µg of amphotericin B per ml of medium. Altogether, stimulated lymphocytes seem to be more susceptible to amphotericin B than some other mammalian cells in vitro, but more resistant to the drug than are many fungi.

Clotrimazole in recommended doses seems to produce serum levels of about 1 µg/ml. The present results do not indicate that concentrations of this magnitude inhibit lymphocyte proliferation.

The mechanisms behind the effects of amphotericin B and clotrimazole on lymphocyte stimulation are unknown. It seems unlikely that any of the antibiotics acts by reacting with the stimulants, as stimulation by many agents was inhibited including the allogenic cell surface in MLC. Effects on the cell membrane similar to those believed to occur when the antibiotics affect fungi are possible but cannot be evaluated from the present results.

ACKNOWLEDGMENT

We thank Ulla Johansson for excellent technical assistance.

LITERATURE CITED

1. Andreoli, T. E. 1973. On the anatomy of amphotericin B-cholesterol pores in lipid bilayer membranes. Kidney Int. **4**:337-345.
2. Bach, M. L., S. Solliday, and M. Stambuk. 1970. Detection of disparity in the mixed leukocyte cultures test: a more rapid assay, p. 643-653. Histocompatibility testing. *In* P. Terasaki (ed.), Proc. 4th Int. Histocompatibility Workshop Conference. Munksgaard, Copenhagen.
3. Bindschadler, D. D., and J. E. Bennett. 1969. A pharmacological guide to the clinical use of amphotericin B. J. Infect. Dis. **120**:427-436.
4. Böyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation. Scand. J. Clin. Lab. Invest. **21** (Suppl.):**27**-50.
5. Coulson, A. S., and G. G. Chalmers. 1964. Separation of visible lymphocytes from human blood. Lancet **(i)**:468-469.
6. Coulson, A. S., and G. G. Chalmers. 1966. Quantitation of peripheral blood lymphocyte cultures. Nature (London) **209**:378-381.
7. Deuticke, B., M. Kim, and Chr. Zöllner. 1973. The influence of amphotericin B on the permeability of mammalian erythrocytes to nonelectrolytes, anions and cations. Biochim. Biophys. Acta **318**:345-359.
8. Fields, B. T., J. H. Bates, and R. S. Abernathy. 1970. Amphotericin B serum concentrations during therapy. Appl. Microbiol. **19**:955-959.
9. Fields, B. T., J. H. Bates, and R. S. Abernathy. 1971. Effect of rapid intravenous infusion on serum concentrations of amphotericin B. Appl. Microbiol. **22**:615-617.
10. Gottlieb, D., H. E. Carter, J. H. Slonekar, and A. Ammann. 1958. Protection of fungi against polyene antibiotics by sterols. Science **128**:361.
11. Hartzman, R. J., F. H. Bach, G. B. Thurman, and K. W. Sell. 1972. Precipitation of radioactivity labeled samples: a semi-automatic multiple-sample processor. Cell Immunol. **4**:182-186.
12. Hemphill, J. J., Y. F. Herman, and V. M. Young. 1957-8. Comparative antifungal activity of nystatin and amphotericin B in tissue culture for virus propagation. Antibiot. Ann. 961-966.
13. Iwata, K., H. Yamaguchi, and T. Hiratani. 1973. Mode of action of clotrimazole. Sabouraudia **11**:158-166.
14. Kinsky, S. C., J. Avruch, M. Permuth, H. B. Rogers, and

A. A. Schonder. 1962. The lytic effects of polyene antifungal antibiotics on mammalian erythrocytes. Biochem. Biophys. Res. Commun. **9**:503–507.

15. Lampen, J., P. Arnow, and R. Safferman. 1960. Mechanism of protection by sterols against polyene antibiotics. J. Bacteriol. **80**:200–206.
16. Littman, M., M. Pisano, and R. Lancaster. 1958. Antibiot Ann 1957/58, 981. Medical Encyclopedia Inc., New York.
17. McCurdy, D. K., M. Frederic, and J. R. Elkinton. 1968. Renal tubular acidosis due to amphotericin B. N. Engl. J. Med. **268**:124–131.
18. Perlman, D., N. A. Giuffre, and S. A. Brindle. 1961. Use of fungizone® in control of fungi and yeasts in tissue culture. Proc. Soc. Exp. Biol. Med. **106**:880–883.
19. Revillard, J.-P. 1971. Potential and actual clinical applications of *in vitro* tests for cell-mediated immunity, p. 154–201. *In* J.-P Revillard (ed.), Cell-mediated immunity in vitro correlates. S. Karger, University Park, Md.
20. Valdimarsson, H., J. M. Higgs, R. S. Wells, M. Yamakura, J. R. Hobbs, and P. J. L. Holt. 1973. Immune abnormalities associated with chronic mucocutaneous candidiasis. Cell. Immunol. **6**:348–361.