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Comparative Toxicities of Amphotericin B and Its Monomethyl Ester Derivative on Glial Cells in Culture

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Amphotericin B (AmB) is a potent antifungal polyene macrolide antibiotic and is the drug of choice for the treatment of deep-seated mycotic infections. Its use is limited, owing to its nephrotoxicity, and it must be dispersed in deoxycholate for parenteral administration. In contrast, AME (the monomethyl derivative of AmB) is water dispersible, is appreciably less cytotoxic than AmB toward a variety of cell types, and is reportedly active against the acquired immunodeficiency syndrome virus (human immunodeficiency virus type 1). The latter activity has generated interest in AME as an antiviral drug. However, AME is perceived to be neurotoxic, based on the outcome of a human clinical trial of AME as an antifungal drug. AmB is not regarded as neurotoxic, presumably because any neurotoxicity in vivo is precluded by its nephrotoxicity. It was important, therefore, to determine the potential for neurotoxicity of the two agents in comparative tests, assessing the effects of their direct action against neural cells in culture. Rat cortical cells, comprising astrocytes and oligodendrocytes, were used. AME was at least 10 times less toxic than AmB and equally less toxic against several other nonneural cell types also included in these tests. Equally important, AmB disrupted the myelin sheath in these cultures, and it inhibited its generation. AME did not, even at a concentration 10 times greater than the toxic concentration of AmB. AmB is, therefore, potentially more neurotoxic than AME, contrary to current perception. AME is effective as an antifungal and antiviral drug at a concentration far below its toxic concentration for neural cells. Also, AME does not cross the blood-brain barrier appreciably, so that a therapeutic level in blood can be expected without encountering neurotoxicity.

Amphotericin B (AmB), as the sodium deoxycholate complex (Fungizone), is the drug of choice for the treatment of deep-seated mycotic infections. The therapeutic index for this drug is relatively low, with multiple, although rare, adverse reactions to the nervous system. Alterations in hearing and vision, headaches, convulsions, and drowsiness, etc., have long been seen as adverse neurological side effects of parenteral AmB therapy (20, 23). Symptoms of major neuropsychiatric disorders after intravenous therapy have also been associated with myelin degeneration seen in peripheral nerve sections (8). Intrathecal AmB therapy, particularly, has been associated with acute febrile meningeal reactions (9), toxic delirium and electroencephalograph abnormalities (18, 21, 22), and myelopathy (3, 4).

The methyl ester derivative of AmB (AME) has a similar spectrum of action against fungal infections (1). Moreover, studies of acute toxicity in animals indicated that AME is significantly less toxic than AmB.

There is an urgent need for new antiviral drugs that are effective against the acquired immunodeficiency syndrome. Both AME and AmB are active against human immunodeficiency virus type 1 and against virus-infected cells (19; O. J. Plescia, D. Pontani, C. P. Schaffner, D. Sun, and P. Saran, Proc. 3rd Int. Conf. AIDS, p. 47, 1987) and therefore warrant serious consideration in the treatment of the acquired immunodeficiency syndrome. Of the two, AME has distinct advantages. It is water dispersible, it is generally 10-fold less cytotoxic against a variety of cell types, and it is also an immunomodulating agent capable of boosting T-cell-dependent immune responses and receptor-mediated phagocytosis (16).

The question arises as to the relative toxicities of AME and AmB on the nervous system. Neuropsychiatric aberrations in patients treated experimentally with AME have been reported (11). Neurohistopathological examination of certain patients who died of their infection revealed diffuse leukoencephalopathy consisting of gliosis with proliferation of gemistocytic astrocytes, pallor of the myelin, and accumulation of macrophages (7, 10). These adverse reactions have been attributed to AME directly or, variously, the effects of metabolites or contaminants in the AME preparations that were used. After total body irradiation and intravenous AmB therapy, similar leukoencephalopathy was also seen in a patient exhibiting akinetic mutism (5).

The ability to culture oligodendrocytes and astrocytes provided an ideal opportunity to examine the direct effects of these two drugs on glial cells as an index of their potential toxicity for neural cells. We could also assess the toxicity of these drugs in terms of their action in disrupting myelin and preventing myelin sheath formation. In this communication, we report the results of such a study, which suggest that AME should be significantly less neurotoxic than AmB in vivo.

MATERIALS AND METHODS

Polyene macrolide antibiotics. AmB as Fungizone (deoxycholate formulation) was purchased from GIBCO Laboratories. Although purchased from GIBCO, this preparation was manufactured by E. R. Squibb & Sons and was of clinical quality. Such preparations are recognized to be free of endotoxins. A stock solution (1 mg/ml of H_2O) was prepared and diluted as desired in tissue culture medium prior to use.

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AME was prepared as the free base by K. Palmer (Division of Laboratories, New Jersey State Department of Health). This preparation contained less than 0.1% of original AmB, and its content of the monomethyl ester was greater than 90% of the methylated derivatives, based on analysis by high-performance liquid chromatography by the procedure of Mechlinski and Schaffner (14). We did not test it for pyrogenicity, but it was presumably free of endotoxin since it was derived from chromatographically pure AmB and it was relatively noncytotoxic in culture against a variety of cell types. A stock solution (1 mg/ml of dimethyl sulfoxide) was prepared and diluted as desired in tissue culture medium prior to use.

Cell lines and antibodies. CTLL-2, an interleukin-2-dependent cell line; BCL-9, a B-cell growth factor-dependent cell line; the T98G primary hepatocyte line; and the K562 line were all purchased from the American Type Culture Collection. David Axelrod (Waksman Institute) kindly provided the 3T3 cell line, the NEJ mutant substrain, and the MCT revertant mutant line. David Goecke (University of Medicine and Dentistry of New Jersey) kindly provided the U-937 macrophagelike cell line. The H-9 cell line, a lymphoblastic leukemic line of cells commonly used as targets of human immunodeficiency virus type 1, was obtained through D. Pontani (New Jersey State Department of Health). Monoclonal antibody A2B5 was obtained from the American Type Culture Collection, monoclonal antigalactocerebroside (anti-GC) was obtained from B. Ranscht (La Jolla Cancer Research Foundation), and polyclonal rabbit anti-glial fibrillary acidic protein (anti-GFAP) was a gift of L. Eng (Palo Alto Veterans Affairs Medical Center). The peripheral blood lymphocytes were from blood of normal volunteers and were recovered by gradient centrifugation in a density gradient of Ficoll-Paque (Pharmacia).

Cell substrates. Glass cover slips (12-mm diameter) were coated with either poly-L-lysine (10 μg/ml; Sigma Chemical Co.), laminin (10 μg/ml; Bethesda Research Laboratories, Inc.), rat tail collagen (Bornstein), or a monolayer of cortical astrocytes prior to the addition of dissociated hypothalmic cells.

Preparation of glial cultures. Astrocytes were prepared by a modification of the method described by Lu et al. (13). The cerebral cortex was dissected from the brains of newborn rats 1 to 3 days postnatally, and the meninges was stripped away as much as possible. The remaining tissue was aseptically sectioned with a scalpel into small pieces, trypsin treated for 20 min, and triturated through a Pasteur pipette, causing dissociation of the cells. Following the suspension of these cells, they were pelleted, suspended in Dulbecco minimal essential medium-fetal calf serum (FCS) culture medium, and plated in Falcon tissue culture flasks (75-cm^2 surface area). One-half of the medium was replaced every 3 days. Once the cells grew to confluence (10 to 11 days), the flasks were shaken overnight on a rotary shaker so as to enable the recovery of nonadherent cells.

Nonadherent cells, consisting of neurons and glial cells and glial progenitor cells of the 02A lineage, were pelleted, resuspended in culture medium, and replated onto 12-mm glass cover slips coated as described above. Each cover slip, with a density of 70×10^3 to 80×10^3 cells, was placed in a well of a 24-well tissue culture plate (Falcon). Cultures were maintained for 1 to 3 days at 37°C with 5% CO_2, after which the cells were characterized for viability and cell type.

Then the drugs were diluted in the appropriate media so that the addition of 0.1 ml of medium gave the desired final concentration of the drug. The results are based on a general analysis of the morphological condition of the observed cells. In addition, the total cell count, over a set number of random fields, was determined so as to compare the possible cytotoxic effects of the drugs during the entire culture period.

Generation of myelin in vitro. Formation of peripheral myelin in vitro was achieved by a modification of the method of Ranscht et al. (17). Briefly, dissociated rat dorsal root ganglion neurons were established in culture free of nonneuronal cells and later repopulated with a pure population of Schwann cells. The dorsal root ganglion neurons from rat embryos were dissected into L15 medium and trypsinized to cause dissociation of cells. After being washed, the cells were plated onto collagen-coated glass cover slips, at between 2,000 and 8,000 cells per cover slip. The elimination of nonneuronal cells was accomplished by successive treatments with fluorodeoxyuridine for 48 h at 10 μM, commencing 1 day after plating. After the final treatment, the cells were maintained for 1 week before the addition of the Schwann cells.

The Schwann cells were obtained from explant cultures of rat embryo dorsal root ganglion neurons free of fibroblasts, as described by Bunge and Bunge (2). Prior to the use of the Schwann cells, neuronal cell bodies were excised. Following treatment with collagenase and trypsin, the single-cell suspension, at 10,000 cells, was seeded onto the above-described neuronal cell network. After attachment, the medium was changed to include 20% FCS and ascorbic acid (75 μg/ml). Generally, myelin segments could be discerned by day 10 and continued to be produced throughout the course of the culture (4 to 5 weeks). The culture medium was renewed generally every 4 to 7 days.

The cultured myelin segments under study were fixed overnight at 4°C with 4% paraformaldehyde and then for 2 h with 0.08% osmium tetroxide. Following dehydration with alcohol at 70%, the cultures were stained with 0.4% Sudan black for 1 h. Phase-contrast and bright-field microscopy were then used to examine the cultures.

Assay for cell permeability. The test cells were treated with filtered trypan blue solution (GIBCO; 1% stock solution filtered three times through a 44-μm [pore size] Falcon filter and diluted to a final concentration of 0.04%). At least 100 cells were counted per field in a standard Levy counting chamber. Viability is expressed as the percentage of cells that were not stained. All values represent the average of at least duplicate counts of duplicate samples.

RESULTS

Comparative cytotoxicity. AME and AmB were compared initially in terms of acute toxicity against several different cell lines. Cells were exposed to these polyenes for 1 h without FCS, after which they were examined for viability. The results (Table 1) represent the percentages of dead cells, including cells lost as a result of lysis and the trypan blue-stained cells. It is apparent that AME was at least 10-fold less cytotoxic than AmB in every cell line tested.

Next, we examined the cytotoxicities of AME and AmB in terms of proliferation of cells in culture during growth in log phase. Test cells were treated with both drugs at several concentrations for 1 h at 37°C without FCS, after which FCS was added and the cell cultures were incubated at 37°C for 4 to 6 days. Cultures of untreated cells were included as controls and provided the base-line value of normal cell proliferation. The results (Table 2) are given as the percent decreases in the number of cells, based on the number of

TABLE 1. Comparative cytotoxicity of AmB and AME against different nonneural cell lines in culture in terms of changes in cell membrane permeability

Cells	% Trypan blue-stained cells[a]					
	Control (no drug)	AmB (μg/ml)			AME (μg/ml)	
		0.1	1	10	1	10	100
Peripheral blood lymphocytes	2	3	7	11	1	3	4
H-9	2	2	5	18	2	2	5
K562	3	1	3	17	3	2	3
T98G	7	6	9	21	7	5	9
CTLL-2	5	4	8	14	6	6	8
BCL-9	8	8	9	23	6	7	10

[a] Acute cytotoxicity is reflected by a change in cell membrane permeability to trypan blue following a 1-h exposure of cells to the polyenes without FCS. The values take into account the loss of cells due to lysis, based on duplicate counts of at least 100 cells per sample of duplicate samples.

cells in control cultures. Cells were counted in a Coulter counter, and the numbers were calculated by integrating the numbers of cells in a distribution of size appropriate for each cell type.

In terms of both acute cytotoxicity (change in membrane permeability) and inhibition of cell proliferation, AME proved to be appreciably less toxic than AmB. This was the case for each of the different types of cells that were treated, including cells of different species. Because neural and nonneural cells differed in their sensitivities to both AME and AmB, we extended this test to different types of brain cells and to cells in different stages of differentiation. Neural

TABLE 2. Comparative cytotoxicity of AmB and AME against different cell lines (neural and nonneural) in culture in terms of inhibition of cell proliferation

Cells	% Inhibition[a]					
	Control (no drug)	AmB (μg/ml)		AME (μg/ml)		
		0.1	1	1	10	100
Nonneural						
Peripheral blood lymphocytes	1	5	22	3	1	5
H-9	6	10	64	2	2	4
K562	8	9	56	1	3	3
U-937	2	8	36	4	2	7
T98G	5	8	43	3	4	10
CTLL-2	2	12	78	0	1	6
BCL-9	2	29	62	6	3	5
3T3	4	9	18	4	1	8
NEJ	5	6	16	2	5	6
MCT	6	18	100	3	7	11
Neural[b]						
Astrocytes	7	21	61	3	7	36
Oligodendrocytes	4	17	57	8	5	55[c]

[a] Growth inhibition was based on the number of viable cells in culture in the presence of 10% FCS. Nonneural cells were pretreated with drug for 1 h without FCS, and viability was determined by exclusion of trypan blue after 4 to 6 days in culture. In the case of the astrocytes and oligodendrocytes, the pretreatment was done in the presence of 10% FCS, followed by a 24-h incubation at 37°C, after which the cells were fixed and stained with fluorescent antibodies, providing identification as well as enumeration of cells. Values represent the average of at least duplicate cultures.

[b] Cultures contained a mixture of astrocytes and oligodendrocytes.

[c] Morphologically, the remaining cells in these cultures appeared normal, in contrast to the cells exposed to AmB at 10 μg/ml.

TABLE 3. Comparative cytotoxicity of AmB and AME against glial cells in culture[a]

Test drug (μg/ml)	Target cell type	% Viable cells[b]
AmB		
10	Oligodendrocytes	37
1	Oligodendrocytes	88
0.1	Oligodendrocytes	94
10	Astrocytes	30
1	Astrocytes	84
0.1	Astrocytes	97
AME		
100	Oligodendrocytes	90
10	Oligodendrocytes	96
1	Oligodendrocytes	96
100	Astrocytes	88
10	Astrocytes	97
1	Astrocytes	95
Control (no drug)	Oligodendrocytes	97
	Astrocytes	95

[a] Cells were obtained from brain tissues of young rats and separated to yield enriched populations of oligodendrocytes and astrocytes. Cultures of these cells, in duplicate, were maintained for 6 days in a 5% CO_2 incubator at 37°C.

[b] Viability was determined by exclusion of trypan blue. Values are averages of duplicate cultures.

cells, enriched in either astrocytes or oligodendrocytes, were incubated on cover slips in the presence of drug for 24 h. Some of these cells were examined for viability in terms of exclusion of trypan blue, and others in replicate cultures were fixed and stained with cell type-specific antibodies, followed by fluorescence-labeled conjugates. The antibodies used were anti-GFAP (a marker of astrocytes), anti-GC (a marker of oligodendrocytes), and antibody A2B5, which recognizes an antigen present on the 02A lineage of oligodendrocytes and astrocytes. Stained cells were examined by fluorescence microscopy over 20 nonoverlapping fields in a crossing pattern. Viable cells were counted in duplicate and independently by separate investigators. The results were virtually identical, so that the data in Table 3 are the averages of these two sets of values. AME at 10 μg/ml showed no toxicity toward either astrocytes or oligodendrocytes. Some toxicity was evident at 100 μg/ml, the viable cells being reduced by about 7%. In contrast, AmB showed some toxicity at 1 μg/ml (more than at 100 μg of AME per ml) and unquestionably was toxic at 10 μg/ml. This differential cytotoxicity between AME and AmB is clearly evident in micrographs of fluorescence-stained glial cells from cultures of those cells exposed to the two drugs (Fig. 1).

Effect on formation and maintenance of myelin sheath in vitro. Experiments were designed to measure the effects of AME and AmB on both the generation of myelin sheath in culture and the integrity of existing myelin sheath at the beginning of the culture. The results (Table 4) were rather dramatic. AmB, in contrast to AME, virtually completely prevented the development of myelin sheath at 10 μg/ml. Even at 100 μg/ml, AME had little effect, if any. In terms of their effects on existing myelin sheath, AmB caused a rapid complete degeneration of myelin, whereas AME at 100 μg/ml had very little effect (data not shown). The observed cytotoxicity of AmB toward both astrocytes and oligodendrocytes, noted above (Table 3 and Fig. 1), explains its ability to inhibit the development of myelin sheath. Its direct toxic effect on myelin is a separate manifestation of the general extensive cytotoxicity of AmB. Thus, AmB is cyto-

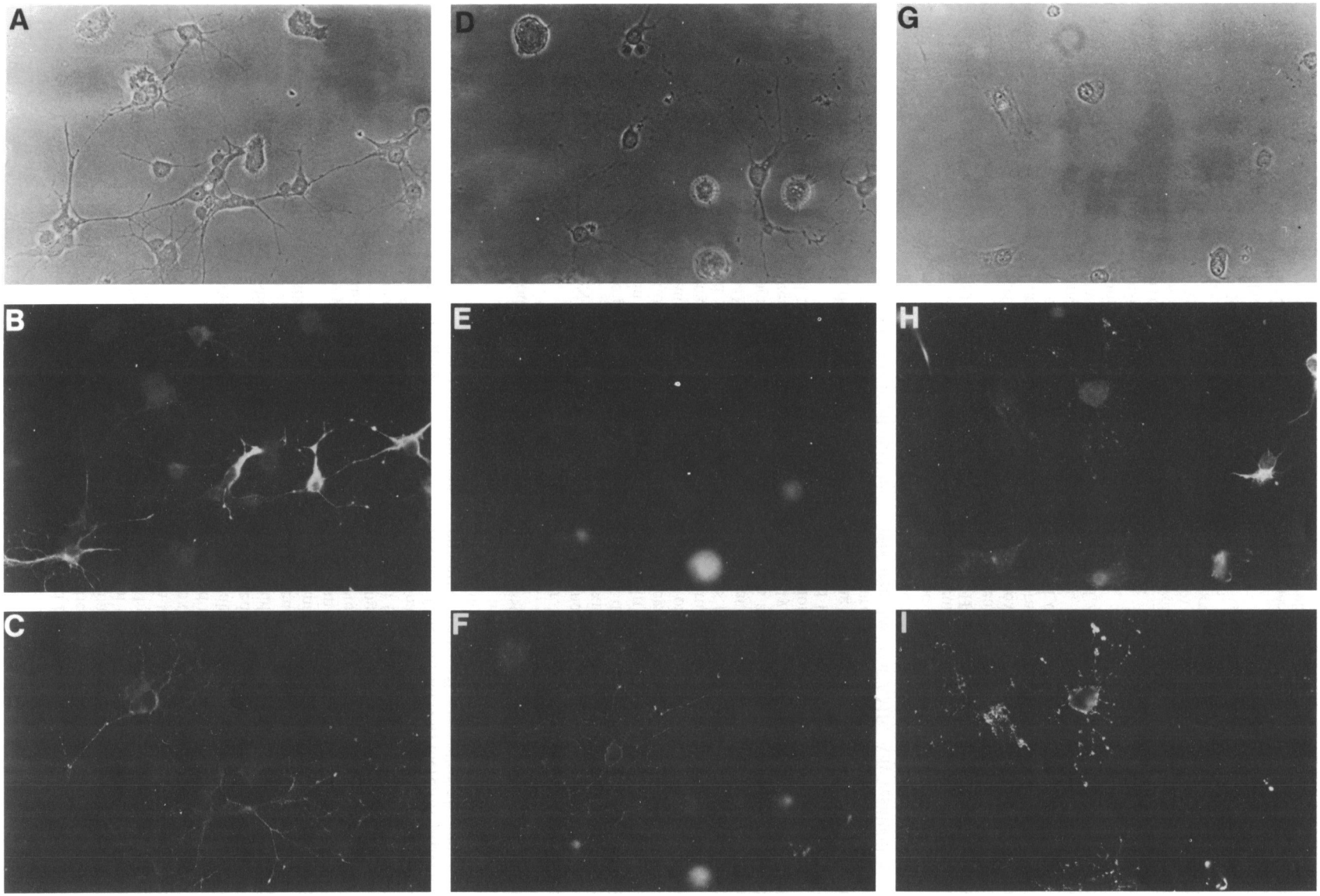

FIG. 1. Effects of AmB and AME on morphology and survival of glial cells in dissociated cultures. Glial progenitors which give rise to either astrocytes or oligodendrocytes were recovered from primary cultures of rat cerebral cortex and plated onto poly-L-lysine-coated glass cover slips. After several days, test substances were added for 24 h and cultures were fixed and stained with anti-GFAP to demonstrate astrocytes or anti-GC to demonstrate oligodendrocytes. (A to C) Control cultures: phase-contrast view of oligodendrocytes and astrocytes (A), same culture viewed with fluorescence optics to reveal GFAP staining on astrocytes (B), and same culture viewed to demonstrate GC staining on oligodendrocytes (C). (D to F) Cultures incubated with 100 μg of AME per ml: phase contrast (D), GFAP (E), and GC (F) showing normal-looking cells. (G to I) Cultures incubated with 10 μg of AmB per ml: phase contrast (G), GFAP (H), and GC (I) showing stunted growth and abnormal morphology.

TABLE 4. Comparative cytotoxicity of AmB and AME against myelin sheath in culture[a]

Test drug (μg/ml)	Result[b]
AmB	
10	No cells visible, no sheath
5	Cells without processes, very little sheath
1	Cells appear normal, sheath only one cell layer thick
AME	
100	Cells normal, sheath less than two cell layers thick
10	Cells normal, sheath three cell layers thick (essentially normal)
1	Cells normal, sheath normal
Control (no drug)	Cells normal, myelin sheath matrix determined to be three cell layers thick

[a] Neuronal cells from rat brain tissue were cultured for 34 days and fed every 7 days with enriched RPMI medium to establish a developing myelin sheath. Test drug was added at the start of the culture and at each feeding. Cultures were examined 3 and 6 days after the last feeding.

[b] Based on microscopic analysis of cell number, myelin sheath matrix, and cell morphology. Cultures were set up in duplicate.

toxic not only for the cells which produce myelin but also for the myelin itself.

DISCUSSION

Polyene macrolide antibiotics are generally relatively cytotoxic and are therefore limited in their therapeutic use. AmB is nevertheless the drug of choice in the treatment of deep-seated mycotic infections because of its potent antifungal activity. AME retains the antifungal activity of AmB, but it is appreciably less cytotoxic and it is water dispersible. Recent reports of the activity of AME against human immunodeficiency virus type 1, the etiologic agent of the acquired immunodeficiency syndrome, has generated renewed interest in AME (16, 19; Plescia et al., Proc. 3rd Int. Conf. AIDS).

AME was tested as an antifungal drug in a human clinical trial some time ago (7). Some patients in this trial showed evidence of leukoencephalopathy, which was attributed to the AME. Although the evidence linking AME to the observed neurotoxicity was not decisive (10), interest in AME as a drug for the treatment of systemic fungal infections waned, and even now it is regarded as a neurotoxic drug, more so than AmB (6, 7).

This study was therefore undertaken to compare the potential neurotoxicities of AME and AmB by the direct actions of the drugs against glial cells in culture. Their cytotoxicity against a variety of other nonneural cell types was also assessed to ascertain whether neural cells might be more sensitive to the cytotoxic action of these polyenes.

Indeed, cells of nervous system origin are somewhat more vulnerable to the sterol-binding polyene macrolides (Tables 1 and 2), and there is a difference in sensitivity even among various types of glial cells (Table 3). This difference very likely reflects quantitative differences in cholesterol in the membranes of different cell types, since the basic action of these antibiotics is to bind to sterols.

The sensitivity of glial cells to the cytotoxic action of AmB and AME was dose dependent, as expected. What is important is the cytotoxic dose range of each used to assess relative cytotoxicity. The results show a striking difference between AmB and AME. AME was at least 10 times less toxic than AmB (Table 3) and was equally less cytotoxic against a variety of different cell types of different species, as reported here (Tables 1 and 2) and by others (12).

A critical feature of leukoencephalopathy is the demyelination that occurs, so that it was important to determine the potential of AmB and AME to disrupt myelin sheath in culture by direct action. There was a striking difference between them in this test also, AmB being at least 10 times more toxic (data not shown). Equally important, AmB inhibited the generation of myelin sheath in culture by glial cells (Table 4), whereas AME did not. These results are supported by the preliminary findings of an in vivo comparative analysis of AmB and AME for neurotoxicity in rats (Ken Reuhl, personal communication).

Our analysis of the neurotoxicity of AmB and AME was based on the direct action of these drugs on glial cells (astrocytes and oligodendrocytes) and on myelin sheath (including its formation). The latter effects on myelin structure and formation are particularly relevant because of the demyelination associated with leukoencephalopathy. The results of such an analysis therefore provide a measure of the potential neurotoxicities of AmB and AME. Unquestionably, AmB is potentially more neurotoxic than AME. Of course, at sufficiently high concentrations (>100 μg/ml), AME also begins to show evidence of neurotoxicity. In contrast, AmB is neurotoxic at 5 μg/ml. It should be noted, however, that neural cells of rat origin were used in this study and that there might be interspecies differences in neurotoxicities of the test drugs. Nevertheless, nonneural cells of different types and species were included in this study. In every instance, AmB was more cytotoxic than AME regardless of species, so that the observed greater toxicity of AmB against neural cells of rat origin is likely to be general and applicable to humans.

These results therefore contradict the perceptions that AME but not AmB is neurotoxic and that therapeutic doses of AME administered parenterally invariably result in neurotoxicity (7). In a recent report, Ellis et al. (6) restated these conclusions. It should be noted, however, that AmB was not included in their study and there was no information given regarding the actual concentration of AME in the circulation or in neuronal tissue, at any time, as a result of administering repeated doses of AME. Any resultant neurotoxicity might have been due to overdosing.

On the basis of the results reported here, AME is potentially neurotoxic, but at a localized concentration of >100 μg/ml. However, AME does not cross the blood-brain barrier easily (15) so that the level of AME in blood would have to exceed, by far, this value to reach a neurotoxic level in the brain. AME is optimally active as an antifungal and antiviral drug at levels of <10 μg/ml, so that it should be possible, with careful monitoring, to achieve therapeutic levels of AME in blood without inducing neurotoxicity.

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