Effects of Antimicrobial Agents Used for Therapy of CNS Infections on Dissociated Brain Cell Cultures

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ABSTRACT. The prediction, measurement, and monitoring of neurologic toxicity of antibacterial agents is an exceedingly difficult matter. In this study we investigated if *in vitro* exposure of cultured brain cells to antibacterial drugs could predict neurotoxicity in man. Effects of antibiotics used for therapy of bacterial CNS infections on growth and differentiation in dissociated rat brain cell cultures were studied over 24 days in culture, the drugs being added from 10 to 17 days in culture, the main differentiation phase of rat CNS cells. Our results demonstrated a reversible inhibition of cerebral sulfate transferase activity ($p < 0.001$ or < 0.01) and to a lesser extent ($p < 0.001$ or NS) of DNA synthesis in brain cell cultures by the highest concentrations studied of amikacin, cefuroxime, and ceftazidime which correspond to peak cerebrospinal fluid values attained by intraventricular therapy in patients. Accumulation of DNA reflects brain cell growth whereas cerebral sulfate transferase activity parallels brain cell differentiation. Our findings indicate that intraventricular therapy could be more toxic with amikacin, cefuroxime, and ceftazidime than with penicillin, chloramphenicol, or ceftriaxone. Thus, this brain cell culture model might become a supplement, complement, or even alternative technique for neurotoxicity assessment of antibiotics with proven or potential value for therapy of CNS infections. (*Pediatr Res* 24: 367–372, 1988)

Abbreviations

BCC, brain cell culture
CSF, cerebrospinal fluid
DIC, days in culture
CST, cerebral sulfate transferase

The neurotoxicity of antibacterial agents is often overlooked or misinterpreted because of its relative infrequency and because its prediction, measurement, and monitoring is extremely difficult (1-3). The neurological side effects of antibiotics include CNS toxicity (convulsions, encephalopathy, neuropsychiatric symptoms), cranial nerve toxicity (optic neuritis, deafness, vertigo), peripheral nerve toxicity (paresthesia, motor weakness, sensory impairment), and neuromuscular blockade. Postmarketing surveillance for such adverse drug reactions has rarely been performed and clinical knowledge is almost exclusively based on

reviews of case reports (1, 2). CNS toxicity is a well-described complication of intraventricular antibiotic therapy for gram-negative bacillary ventriculitis and CSF shunt infections (1, 2, 4-7). Animal toxicity tests bear a relatively poor predictive value for neurotoxicity in man and require the use of a high number of animals (4, 5, 8-10). Therefore, back-shifting of neurotoxicity tests from the human or animal situation to a relatively simple and reproducible system *in vitro* was the principal aim of the present research.

There are many reports presenting evidence of dose-dependent antiproliferative effects of various antibiotics on cultured eucaryotic cells. The cell types studied include human fibroblasts (11), bone marrow cells (12), and dissociated brain cells (13). As *in vitro* correlative of dose-related marrow suppression by chloramphenicol it was found several decades ago that this drug inhibits mitochondrial protein synthesis not only in bacteria but also in proliferating bone marrow cells (14, 15). More recently, the inhibition of cell proliferation has most often been studied for the β-lactam antibiotics on cultured human myeloid precursor and lymphoid cells, the clinical consequences being neutropenia and impairment of cellular and humoral immune responses induced by β-lactams (12, 16-18).

The purpose of our investigation was to monitor the effects of antibiotics on growth and differentiation in dissociated rat BCC which might help to predict human neurotoxicity. Standard and newer antibacterial agents used for meningitis and ventriculitis therapy were studied. The drug concentrations were chosen such as to represent peak values attained in ventricular CSF during intravenous therapy (19-22), and their 5 and 10 times multiples that correspond to the concentration range reached by intraventricular antibiotic administration (23-25).

Potential adverse drug effects on BCC were studied during the essential differentiation phase of rat CNS cells between day 10 and 17 in culture. From day 17 to 24 in culture any regeneration capacity was assumed to become manifest.

MATERIALS AND METHODS

Cultures. Dissociated brain cells were prepared from newborn rats (Sprague-Dawley) as previously described (26) except that no trypsin was used. A 10-ml aliquot of cell suspension (10^5 cells/ml) was seeded into culture dishes that immediately before had been exposed for 1 h to Poly-L-Lysine (Sigma P-1524) and washed for 1 h with sterile water. The medium used was modified Eagle medium (Dulbecco) supplemented with 10% fetal calf serum. This basic culture medium contained no antibiotics and was changed on 3, 6, 8, 10, 13, 15, 17, 20, and 22 DIC at 37° C and 5% CO_2. Each experiment was performed in triplicate. For each test culture with antibiotics added there was a control culture without antibiotics. Cell viability during culture process was regularly assessed by macroscopic and phase contrast microscopic examination.

Addition of antimicrobial agents. The drugs and concentrations tested are listed in Table 1. The pure antibiotics were obtained through the hospital pharmacy and were available as either sodium salt (penicillin, cephalosporins, chloramphenicol) or sulfate (aminoglycosides) to be dissolved in sterile water. Generic names, trade names, and manufacturers of the study drugs were: benzylpenicillin sodium (penicillin G, Hoechst), streptomycin sulfate (Streptothenat, Grünenthal), amikacin sulfate (Amikin, Bristol-Myers), chloramphenicol sodium succinate (Chloromycetin, Parke-Davis), cefuroxime sodium (Zinacef, Glaxo), ceftriaxone sodium (Rocephin, Roche), and ceftazidime sodium (Fortam, Glaxo). Aliquots of concentrated stock solutions were stored at $-20°$ C.

The antimicrobial agents were added to the BCC medium to get the final concentration per ml of medium given in Table 1 on DIC 10, 13, and 15. Antibiotics were mixed with the medium by agitation of the dish. From DIC 17 on, only antibiotic-free medium was used again. Exposure to antibiotic in test BCC was therefore 7 days (DIC 10-17).

Analytical methods. At DIC 10 (days 0-10 control and test BCC without antibiotics), 14 and 17 (days 10-17 exposure to antibiotics in test BCC), and 24 (days 17-24 control and test BCC without antibiotics) the cultured cells were harvested by scraping with a rubber policeman, washed, and centrifuged. The cell sediments were kept frozen at $-20°$ C until assayed within 3 wk. Complete homogenization of brain cells was secured by thawing and addition of 200 µl of homogenization buffer (50 mM $NaH_2 PO_4$, 1 mM EDTA Na_2, Triton X-100 1%, pH 7.0). Total protein was determined by the method of Lowry et al. (27), and total DNA according to Hinegardner (28). Activity of CST was assayed as previously described (29). Accumulation of protein and DNA reflects brain cell growth (26) whereas CST activity parallels brain cell differentiation (29).

Drugs assays. Antibiotic concentrations were measured in culture medium and brain cell lysates of some of the BCC experiments with the aim to determine half-life in medium and penetration into brain cells. The assay methods used were enzyme immunoassay technique for amikacin (30) and high-pressure liquid chromatography for cefuroxime (31), ceftriaxone (32), and ceftazidime (33). Assays of penicillin, streptomycin, and chloramphenicol were not performed.

Statistics. Significance between test and control BCC measurements of protein and DNA content and CST activity was determined using the paired Student's t test on two to four separate experiments performed in triplicate. The results were confirmed by the paired Wilcoxon test (34).

RESULTS

Cell growth and differentiation. Macro- and microscopic examinations indicated regular growth of morphologically normal brain cells. To compare the sensitivity of the present rat BCC model to the previously used mouse BCC model (13), the experiment with the penicillin/streptomycin combination (200 IU/50 µg/ml culture medium) generally used to maintain sterility in cell cultures was first repeated. As shown in Table 2 this drug combination compared to control reduced significantly DNA content at DIC 14 ($p < 0.05$) and CST activity at DIC 17 ($p < 0.001$) as reported before (13).

The lower and intermediate drug concentrations tested (Table 1) had no effect on the different parameters studied (12 experiments in triplicate, data not shown). The results of protein concentration (mg/culture), DNA concentration (µg/culture), and CST activity (pmol/h/mg protein) measurements at DIC 10, 14, 17, and 24 in the six experiments with the highest antibiotic concentrations studied are listed in Tables 2 and 3 as

Table 1. *Antimicrobial agents and concentrations tested*

Drugs	Concentrations (per ml medium)		
Penicillin G (sodium) +	200 IU		
streptomycin (sulfate)	50 µg		
Penicillin G*	20 IU	100 IU	200 IU
Amikacin (sulfate)	10 µg	50 µg	100 µg
Chloramphenicol (sodium succinate)	20 µg	100 µg	200 µg
Cefuroxime (sodium)	20 µg	100 µg	200 µg
Ceftriaxone (sodium)	20 µg	100 µg	200 µg
Ceftazidime (sodium)	20 µg	100 µg	200 µg

* Concentrations of penicillin G given in IU/ml; 20 IU/ml = 11.2 µg/ml, 100 IU/ml = 56 µg/ml; 200 IU/ml = 112 µg/ml.

Table 2. *Effects of highest drug concentrations studied of penicillin/streptomycin, penicillin, amikacin, and chloramphenicol on dissociated BCC (mean values ± SD)*

Antibiotics (per ml medium)	DIC	n*	Protein (mg/culture)			DNA (µg/culture)			CST (pmol/h/mg protein)		
			Control	Test	p†	Control	Test	p†	Control	Test	p
Penicillin (200 IU) +	10	9	0.6 ± 0.2			80 ± 18			6.5 ± 2.0		
streptomycin (50	14	9	0.8 ± 0.1	0.8 ± 0.1	NS	109 ±9	98 ± 10	<0.05	10.5 ± 1.4	10.5 ± 1.8	NS
µg)	17	9	1.0 ± 0.1	0.9 ± 0.2	NS	106 ± 17	104 ± 20	NS	14.1 ± 3.9	7.7 ± 0.9	<0.001
	24	9	1.3 ± 0.1	1.3 ± 0.1	NS	114 ± 9	114 ± 21	NS	14.1 ± 1.9	14.1 ± 1.6	NS
Penicillin (200 IU)	10	12	0.6 ± 0.1			70 ± 12			9.6 ± 0.8		
	14	12	1.2 ± 0.1	1.2 ± 0.1	NS	131 ± 22	126 ± 23	NS	11.3 ± 1.2	11.5 ± 0.7	NS
	17	12	1.2 ± 0.3	1.2 ± 0.3	NS	112 ± 15	113 ± 23	NS	14.0 ± 1.1	13.0 ± 2.3	NS
	24	12	1.6 ± 0.3	1.5 ± 0.2	NS	140 ± 27	126 ± 29	NS	27.6 ± 3.3	27.5 ± 4.1	NS
Amikacin (100 µg)	10	9	0.4 ± 0.2			39 ± 15			4.1 ± 1.4		
	14	9	0.8 ± 0.2	0.8 ± 0.2	NS	71 ± 13	69 ± 19	NS	5.1 ± 0.7	3.8 ± 0.3	<0.001
	17	9	0.9 ± 0.2	1.0 ± 0.1	NS	74 ± 21	87 ± 34	NS	7.7 ± 0.4	5.3 ± 0.7	<0.001
	24	9	1.3 ± 0.4	1.4 ± 0.4	NS	91 ± 22	91 ± 27	NS	9.5 ± 1.5	8.0 ± 1.1	<0.01
Chloramphenicol	10	9	0.3 ± 0.1			27 ± 6			2.6 ± 2.4		
(200 µg)	14	9	1.2 ± 0.2	1.4 ± 0.1	NS	84 ± 8	89 ± 14	NS	10.1 ± 2.5	9.1 ± 1.0	NS
	17	9	0.7 ± 0.1	0.7 ± 0.1	NS	64 ± 18	66 ± 10	NS	13.9 ± 1.9	13.6 ± 1.5	NS
	24	9	0.9 ± 0.2	0.8 ± 0.2	NS	90 ± 24	75 ± 17	NS	14.4 ± 3.0	16.2 ± 1.5	NS

* Number of culture plates analyzed.

† Student's t test between test (with antibiotics) and control cultures (without antibiotics).

mean values ± SD. A statistically significant inhibition of cell growth (protein and DNA synthesis) or an effect on cell differentiation (CST activity) became manifest at exposure to 100 μg/ ml of amikacin, 200 μg/ml of cefuroxime, and 200 μg/ml of ceftazidime. The most pronounced differences compared to control ($p < 0.001$) were found at DIC 14 and 17, whereas at DIC 24 the majority of inhibitory effects had disappeared. In Figure 1 the changes in protein and DNA content, and CST activity during BCC control and test experiments with the two-third generation cephalosporins ceftriaxone and ceftazidime at concentration of 200 μg/ml medium are depicted. The transient decrease of CST activity at DIC 17 observed during exposure to 200 μg/ml of ceftazidime clearly contrasts to control and ceftriaxone experiments.

In Figures 2 and 3 the measured values of DNA content and CST activity in test cultures are expressed in percent of these values as compared to control cultures. The relative effect of the three different drug concentrations of the six antibiotics included in this study are shown at DIC 14, 17, and 24. As stated before, lower and intermediate drug concentrations did not produce any substantial reduction. The statistically significant inhibitory effects of 100 μg/ml of amikacin, 200 μg/ml of cefuroxime, and 200 μg/ml of ceftazidime are also evident in the percent data presentation.

Drug levels. The antibiotic concentrations measured in culture medium showed potency loss over 2 days at 37° C of approximately 10% for amikacin and approximately 50% for the cephalosporins.

Drug concentration measurements in cell lysate revealed for amikacin a cell penetration of approximately 50% at DIC 14, of approximately 80% at DIC 17, and of approximately 60% at DIC 24. Mean values found in cell lysate of experiments with the three amikacin concentrations of 10/50/100 μg/ml culture medium were: DIC 14, 5.2/27.2/48.7 μg/ml; at DIC 17, 7.1/ 41.3/79.0 μg/ml; and at DIC 24, 5.8/30.8/55.3 μg/ml. For the cephalosporins, the great majority (>90%) of drug assays in cell lysates did not detect any measurable concentration, the lower limit of detection being 1.0 μg/ml.

DISCUSSION

The results of the present study demonstrated reversible inhibition of CST activity and to a lesser extent of DNA synthesis in BCC by the highest concentrations studied of amikacin, cefuroxime, and ceftazidime. Accumulation of protein and DNA reflects brain cell growth (26) whereas CST activity parallels brain cell

differentiation (29). The antibacterial agents were added to BCC from 10 to 17 DIC which corresponds to the essential differentiation phase of rat CNS cells (26). Dissociated BCC consist to a major part of oligodendrocytes besides astrocytes and neurons
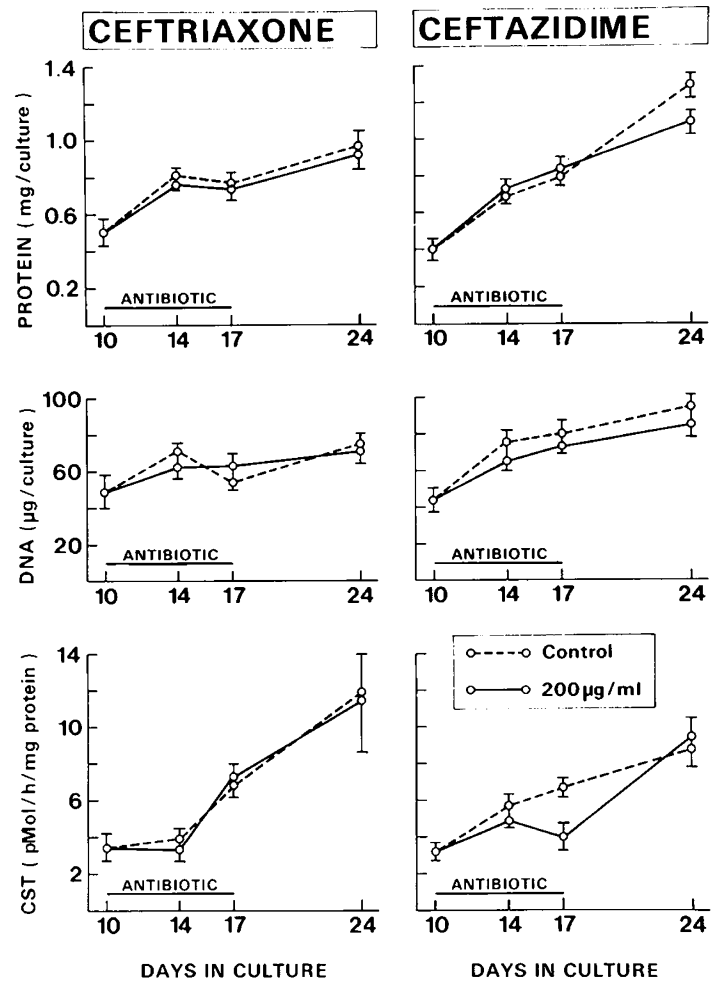

Fig. 1. Changes in total protein and DNA concentrations (per culture) and CST activity (per mg protein) during control (no antibiotics) and test BCC experiments with ceftriaxone and ceftazidime: 200 μg/ml of medium from day 10 to 17. Mean values ± SEM, $n = 9$.

Table 3. *Effects of highest drug concentrations studied of cefuroxime, ceftriaxone, and ceftazidime on dissociated BCC (mean values ± SD)*

Antibiotics (per ml medium)	DIC	n*	Protein (mg/culture)			DNA (μg/culture)			CST (pmol/h/mg protein)		
			Control	Test	p†	Control	Test	p†	Control	Test	p†
Cefuroxime	10	6	0.4 ± 0.1			63 ± 14			7.5 ± 2.2		
(200 μg)	14	6	0.9 ± 0.2	0.9 ± 0.2	NS	103 ± 12	81 ± 19	<0.01	8.1 ± 0.9	6.3 ± 0.4	<0.01
	17	6	0.8 ± 0.2	0.9 ± 0.1	NS	74 ± 40	54 ± 15	NS	11.1 ± 0.6	9.3 ± 1.3	NS
	24	6	1.2 ± 0.1	1.2 ± 0.1	NS	105 ± 20	100 ± 17	NS	16.8 ± 3.6	15.3 ± 3.6	NS
Ceftriaxone	10	9	0.5 ± 0.2			49 ± 18			3.4 ± 2.2		
(200 μg)	14	9	0.8 ± 0.1	0.8 ± 0.1	NS	70 ± 7	63 ± 13	NS	3.9 ± 1.0	3.3 ± 1.9	NS
	17	9	0.8 ± 0.1	0.8 ± 0.2	NS	54 ± 11	63 ± 20	NS	6.9 ± 2.2	7.2 ± 2.4	NS
	24	9	1.0 ± 0.3	0.9 ± 0.3	NS	74 ± 16	73 ± 22	NS	11.9 ± 6.2	11.6 ± 9.1	NS
Ceftazidime	10	9	0.4 ± 0.1			44 ± 19			3.2 ± 0.7		
(200 μg)	14	9	0.7 ± 0.1	0.7 ± 0.1	NS	75 ± 23	65 ± 15	NS	5.7 ± 1.6	4.9 ± 0.8	NS
	17	9	0.8 ± 0.1	0.8 ± 0.2	NS	80 ± 11	74 ± 11	NS	6.7 ± 1.1	4.0 ± 0.9	<0.001
	24	9	1.3 ± 0.2	1.1 ± 0.2	NS	94 ± 20	85 ± 22	NS	8.8 ± 2.7	9.4 ± 2.8	NS

* Number of culture plates analyzed.

† Student's *t* test between test (with antibiotics) and control cultures (without antibiotics).
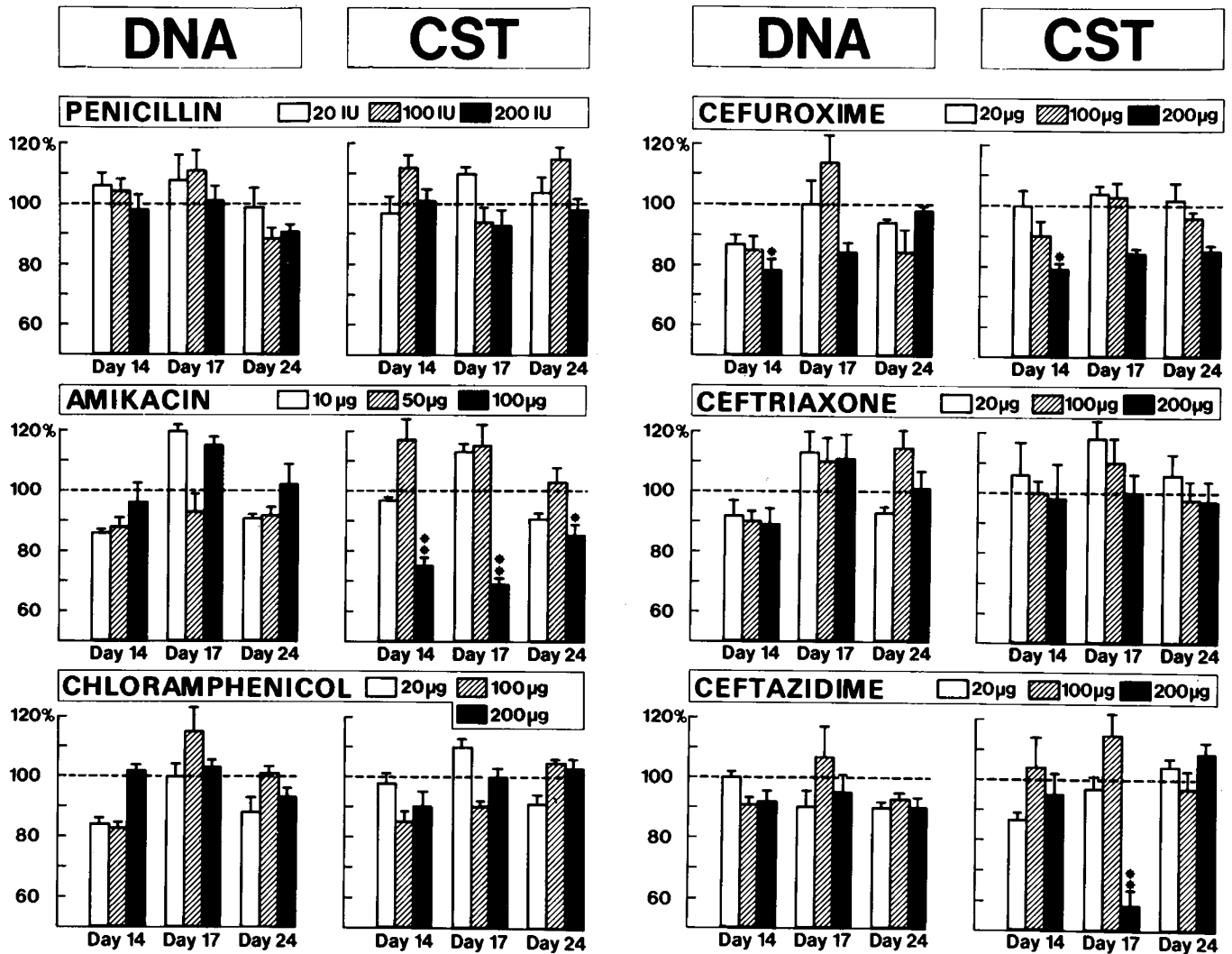
Figs. 2 and 3. Effects of three different drug concentrations (per ml of medium) of the six study antibiotics on DNA content and CST activity in BCC. Mean values of six to nine experiments with antibiotics added from day 10 to 17 are expressed in percent of corresponding control cultures without any antibiotics. *Vertical bars* represent SEM; $* p < 0.01$; $** p < 0.001$.

(35). Our data suggest that certain antibiotics exhibit a dose-dependent suppressive effect on differentiation and to a lesser extent on proliferation of oligodendrocytes in rat BCC. Both of these drug actions showed complete regeneration during the antibiotic-free period from 17 to 24 DIC and did not affect cell viability. These experimental data could indicate that the drugs principally interact with enzyme activity. This interpretation of drug mechanism is consistent with recent publications demonstrating dose-dependent inhibition of the replicative DNA polymerase enzyme in eucaryotic cells by β-lactam antibiotics (17, 18). Interestingly, the same research group has established a rank order of β-lactams for their inhibitory capacity on bone marrow cultures (12, 18) with good correlation to clinical induction of neutropenia. The suppressive effects of the β-lactams studied in our BCC model followed the same rank order: ceftazidme > cefuroxime > ceftriaxone and penicillin. It is possible that the negative drug effects on cell proliferation and differentiation are more pronounced in cultured cell populations more rapidly dividing than rat BCC.

The inhibitory potency of β-lactams on *in vitro* granulopoiesis seems to be substantially enhanced by drug degradation (16). However, the convulsant effect, like the antimicrobial effect, depends on the presence of the intact β-lactam ring (2, 36). It has been shown in animal experiments that penicillinase-treated penicillin G is no more epileptogenic (37).

The most pronounced effect in our experiments was the inhibition of CST activity by amikacin in the concentration of 100 μg/ml medium. The primary mode of bactericidal action of aminoglycosides is due to their specific attachment to ribosomes and subsequent disruption of the translation mechanism of the cell (38, 39). It has been speculated that these mechanisms also account for the use of aminoglycosides as inhibitors of eucaryotic cells in the development of interspecific cloning vectors (39). Reduced CST activity could reflect impaired CST synthesis due to ribosomal interaction of amikacin in the BCC model.

Antibiotic concentration measurements in culture medium confirmed the anticipated potency loss within 2 days of approximately 10% for amikacin and approximately 50% for the cephalosporin compounds. Attempts to assess drug cell penetration in this *in vitro* model were based on antibiotic determination in cell lysates (40). The assumed high cell penetration of amikacin was confirmed whereas the cephalosporin data proved to be too variable to allow any conclusion concerning brain cell-associated antibiotic. Penetration of antibiotics into eucaryotic cells is extremely complex and depends on a variety of factors such as lipid solubility, protein binding, molecular size, and structure of both, cell membrane and intracellular organelles (40, 41). In addition to limited brain cell penetration also binding to cellular structures and potency loss could have been responsible for the in >90% nondetectable cephalosporin concentrations in the cell

lysates. A possible relation between cell penetration and suppressive effects of antibiotics in this BCC model is not assessable by our data.

The limited clinical knowledge on neurotoxicity of antimicrobial agents (1–3) and difficulties in extrapolating *in vivo* animal models (4, 5, 8–10) prompted the present *in vitro* research. The advantages of *in vitro* assessment are better reproducibility and smaller number of variables. Prognosis of gram-negative bacillary ventriculitis and CSF shunt infections remains relatively poor. The major therapeutic problems of these CNS infections are antibiotic resistance of involved, often nosocomially acquired, gram-negative and gram-positive pathogens, and limited drug penetration into CSF because of narrow therapeutic drug index or only low-grade meningeal inflammation (6, 7). Therefore, to produce larger antibiotic concentrations in CSF direct drug inoculation is often recommended in addition to systemic therapy (6, 7, 23, 24, 42). Neurotoxicity represents a potential hazard for intraventricular antibiotic administration and has been clinically and experimentally repeatedly observed (1, 2, 4, 23–25). Histopathologic changes in rabbits due to gentamicin included ependymal and brain cell damages (4, 5). The highest antibiotic concentrations studied in our experiments correspond to the peak values in CSF attained by intraventricular administration in patients (6, 7, 23, 24, 42). To imitate the clinical situation of intraventricular therapy the study drugs were taken from regular injectable drug vials as sodium or sulfate salts. Therefore, our experiments do not allow to exclude effects of the salt components on BCC.

We conclude that the *in vitro* phenomena observed in these studies could have some predictive value for clinical neurotoxicity. The no-effect levels may be of at least equal significance as the toxic-effect levels. Simple extrapolation of results of toxic effects from *in vitro* to *in vivo* situation and across species is obviously unrealistic (9). The major limitations of our *in vitro* test system for drug neurotoxicity are elimination of blood-brain barrier, lack of metabolic and elimination functions, and absence of inflammation. We suggest that the clinical use of antimicrobial agents that produce significant inhibition of cell growth and differentiation in this BCC model implies close monitoring for potential neurotoxicity, especially during intraventricular drug administration.

Inclusion of histopathologic and immunofluorescent studies (35), analysis for degradation products of antibiotics (16, 18), and evaluation of the salt component of antibiotic might add to the predictive value of our model. These studies are part of our planned further investigations.

Implications. Our findings indicate that intraventricular therapy could produce more neurotoxicity with amikacin, cefuroxime, and ceftazidime than with penicillin, chloramphenicol, or ceftriaxone. So far, clinical experience with intraventricular amikacin, cefuroxime, ceftazidime, and also ceftriaxone administration is very scant and, to our knowledge, neurologic side effects have not been reported. Careful monitoring for neurotoxic signs and symptoms is mandatory in such patients. We suggest that the sensible use of our BCC model could represent an important component in the early assessment for neurotoxicity of antimicrobial agents with proven or potential value for therapy of CNS infections.

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