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Late Treatment with Polyene Antibiotics Can Prolong the Survival Time of Scrapie-Infected Animals

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Amphotericin B (AmB) is one of the few drugs able to prolong survival times in experimental scrapie and delays the accumulation of PrPres, a specific marker of this disease in the brain in vivo. Previous reports showed that the AmB effect is observed only if the drug is administered around the time of infection. In the present study, intracerebrally infected mice were treated with AmB or one of its derivatives, MS-8209, between 80 and 140 days postinoculation. We observed an increased incubation time and a delay in PrPres accumulation and glial fibrillary acidic protein gene expression. Treatment starting at 80 days postinoculation was as efficient as long-term treatment starting the day of inoculation. Our results indicate that polyene antibiotics may interfere, throughout the course of the experimental disease, with the propagation of the scrapie agent.

The transmissible spongiform encephalopathies (TSE) are characterized by a long incubation period and a rapid fatal outcome after the onset of clinical signs. The neuropathological characteristics, including spongiosis and gliosis, are found both in the natural disease and in experimentally infected rodents, ruminants, and primates (9). During experimental infection, a posttranslational (29) accumulation in the brain of a pathological isoform (PrPres) (5) of a host-encoded protein (PrPsen) (14) is observed concomitantly with an increase of infectivity (21). PrPres accumulation occurs in different regions of the central nervous system, depending on both the strain of TSE agent inoculated and the host genotype (9). In addition, activation of astrocytes leads to overexpression of the glial fibrillary acidic protein (GFAP) gene (18, 24). PrPres accumulation and GFAP gene overexpression are two molecular hallmarks of experimental scrapie.

The polyene macrolide antibiotic amphotericin B (AmB) is a widely used antifungal agent whose action seems to occur via an interaction with the ergosterols (4). AmB has been shown to prolong survival time in Syrian hamsters infected with the 263K scrapie strain (3, 32). Furthermore, AmB administration reduces PrPres accumulation in scrapie-infected animals (15, 26, 39), suggesting that survival time is correlated to the amount of PrPres in the brain. These biological effects of AmB were observed only in one experimental model, the Syrian hamster infected with the 263K scrapie strain. To achieve a significant effect, the drug was administered at an early time during the disease course. The effects of polyene antibiotics on intraperitoneally (i.p.) infected hamsters suggested that these drugs could have an effect subsequent to initial infection, perhaps by inhibiting the spread of the TSE agent along the peripheral nerves (31). In fact, AmB did not increase survival times in hamster infected by the intracerebral (i.c.) or intraperitoneal (i.p.) route when AmB was given after TSE agent started to multiply in the brain (31).

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The lack of effect of AmB administration during the preclinical or clinical stage of the disease and the restriction of the effect to 263K-infected hamsters could be explained by low penetration of the drug into the central nervous system. Indeed, the cerebral concentration of AmB reached only 0.3 μg/g when Syrian hamsters were treated i.p. with 1 mg of AmB per kg of body weight (12), and AmB was undetectable in rat brain following i.p. administration of a similar dose (28). Use of AmB and a less toxic derivative, MS-8209, allowed us previously to observe prolongation of survival time in C57BL/6 mice infected i.c. or i.p. with the C506M3 scrapie strain (15). Thus, the use of these drugs in late treatment could lead to a prolonged survival time.

The present experiments were designed to study the effects of higher doses of polyene antibiotics, AmB and MS-8209, in the late stage of experimental scrapie. In C57BL/6 mice infected i.c. with the C506M3 scrapie strain, increases in infectivity titer and PrPres accumulation are detected in brain around 60 to 80 days postinoculation (dpi). Histopathological lesions appear around 110 dpi, and neurological symptoms appear at 140 dpi. As treatment schedules, we chose different periods of time after the appearance of PrPres accumulation and TSE agent multiplication in the brain. We observed both a delay in PrPres accumulation and an increase in survival time of infected animals after administration of polyene antibiotics. The increase in survival time was correlated with the dosages used even in very late treatment.

MATERIALS AND METHODS

Animals. Mice used in the study were purchased from IFFA-CREDO (St. Germain d'Arbresle, France). C57BL/6 females were 4 weeks old at the time of inoculation. Animals were sacrificed by cervical column disruption. The central nervous system, including cerebellum, was dissected, rapidly frozen in liquid nitrogen, and kept at −80°C until use. Groups of three animals were sacrificed for molecular analysis. Animals were regularly monitored for clinical signs. Survival times were statistically analyzed by a nonparametric (Mann-Whitney) test.

Scrapie strain. The C506M3 scrapie strain was a gift from P. Brown and D. C. Gajdusek (National Institutes of Health, Bethesda, Md.) and was passaged seven times in our laboratory. The scrapie inoculum had been titrated in vivo by limiting dilution 50% lethal dose (LD_{50}) assay. Twenty microliters of 1% (wt/vol) homogenate, 1.6×10^8 LD_{50}, was injected into the right cerebral hemisphere.

Drugs. AmB was obtained as deoxycholate salt (Fungizone, Squibb-Meyer). MS-8209 is the *N*-methyl glucamine (NMG) salt of 1-deoxy-1-amino-4,6,*O*-benzylidine-D-fructosyl-AmB (kindly provided by Mayoly-Spindler Laboratories, Chatou, France). The maximal tolerated dosages for chronic i.p. administration of AmB and MS-8209 are 7 and 30 mg/kg, respectively, in C57BL/6 mice (data not shown). Drugs were resuspended in a 5% (wt/vol) glucose sterile solution. Animals were treated 6 days a week by the i.p. route. Controls consisted of untreated and solvent NMG (Sigma)-treated infected mice.

Estimation of infectious titer. Estimations of infectious titers were done by injecting 20 μl of 20% brain homogenates in recipient mice. Survival time was noted and compared to a standard curve of incubation time versus infectious titer.

Immunization. New Zealand rabbits were immunized with a 20-mer synthetic peptide (G-Q-G-G-G-T-H-S-Q-W-N-K-P-S-K-P-K-T-N-Y) corresponding to amino acids 93 to 108 in the human PrP sequence. This peptide was linked to keyhole limpet hemacyanin via its N-terminal tail (Neosystem, Strasbourg, France). Primary immunization was carried out with 150 μg of the coupled peptide mixed in complete Freund's adjuvant. Four boosts with 150 μg of the coupled peptide were done in incomplete Freund's adjuvant at 3-week intervals, and blood was collected from a marginal ear vein. In a Western blot assay, the antiserum reacted with PrPres extracted from brains of mice infected with the C506M3 strain and from brains of hamsters infected with the 263K strain. This antiserum, called 007JB, was used for Western blotting at a dilution of 1:5,000.

Protein. A 50-μl aliquot of 20% (wt/vol) brain homogenate was digested by proteinase K (PK; 20 μg/ml; Sigma) for 1 h at 37°C in order to remove all the cellular PrP. Samples were boiled in 200 μl of denaturation buffer (Tris-glycine [pH 7.2], 2% sodium dodecyl sulfate [SDS], 2% β-mercaptoethanol, 5% sucrose) for 5 min. Samples were then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on a 12% polyacrylamide separation gel, and proteins were transferred onto nitrocellulose membranes (0.45-μm pore size; Hoefer). After blocking in 5% (wt/vol) milk–0.1% Tween 20–0.1% NaN_3 in phosphate-buffered saline, membranes were incubated with a polyclonal anti-mouse PrP antibody (007JB, 1:5,000). After washes in phosphate-buffered saline–0.1% Tween, membranes were incubated with peroxidase-conjugated anti-immunoglobulin goat antibody (Southern Biotechnologies). Immunodetection was carried out with an ECL (enhanced chemiluminescence) kit (Amersham), and signals were quantified with a radio imager (PhosphoImager; Bio-Rad).

Northern blotting. Total RNA was extracted and size fractionated on a 0.6 M formaldehyde–1% agarose gel. RNA was blotted onto a nylon membrane (Schleicher & Schuell) by using the Vacugene system (Pharmacia, LKB) and cross-linked by 260-nm UV irradiation. Blots were hybridized with [α-^{32}P]dCTP (110 MBq/mmol; Amersham)-labeled probes (10^8 cpm/μg). Probes were as follows: (i) a 1.3-kb *Pst*I fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (gift from P. Fort, Université de Montpellier, Montpellier, France); (ii) a 1.1-kb *Hin*dIII fragment GFAP probe (kindly provided by N. J. Cowan, New York University, New York, N.Y.); and (iii) a 1.8-kb *Hin*dIII fragment PrP probe (gift from D. Westaway, University of California, San Francisco). Stringent washing procedures included three successive 20-min incubations at 42°C in 0.1% SDS and 2×, 1×, and 0.1× SSPE (1× SSPE is NaCl [174 g/liter] plus Na_2HPO_4 [27.6 g/liter], pH 7.4). Signals were quantified with a radio imager (Bio-Rad) and normalized by comparison with the GAPDH signals.

RESULTS

Late administration of polyene antibiotics prolongs survival time. To test the effects of polyene antibiotics during late times of experimental scrapie, we administered AmB or MS-8209 to i.c.-infected mice from 90 to 120 dpi, a period which has been proven to prolong incubation times when administration begins at the day of inoculation (data not shown). Following administration of 2.5 mg of MS-8209 or AmB per kg, we observed an increase in survival time in treated mice (Table 1). As previously observed, the duration of the clinical phase and clinical symptoms were identical in all groups (15). Thus, the increased survival time was due to a prolonged incubation phase. This is the first report of a prolonged incubation time when drug administration was initiated so late after i.c. infection with scrapie.

Following AmB or MS-8209 administration from 90 to 120 dpi, PrPres levels were lower than in control groups (Fig. 1). Similar results have been obtained in three distinct experiments. AmB treatment was more efficient at reducing PrPres levels, since the same effect was observed at 25 mg of MS-8209 per kg and only 2.5 mg of AmB per kg. Similar differences in efficiency were noted previously for long-term treatment starting at the day of inoculation (15).

TABLE 1. Survival times of mice treated with AmB or MS-8209 from 90 to 120 dpi[a]

Expt	Drug	Survival time	Delay (*P* value)
	None	159.8 ± 2.5 (4)	
A	AmB	169.7 ± 3.1 (5)	9.95 (NS)
	MS-8209	176 ± 4.4 (5)	16.2 (0.01)
	None	169.4 ± 4.7 (11)	
	Solvent (NMG)	166.1 ± 2.5 (7)	−3.3 (NS)
B	AmB	179.9 ± 6.3 (8)	13.8 (0.01)
	MS-8209	178.7 ± 4.2 (7)	12.6 (0.01)
	MS-8209 (25 mg/kg)	190.6 ± 9.1 (7)	24.5 (0.01)

[a] Survival times are expressed in days ± standard error followed by the effective in parentheses. Dosage was 2.5 mg/kg except as noted. In both experiments, mice were inoculated with 1.6×10^8 LD_{50}. Delays (in days) and statistical significance were calculated from data for the untreated group for experiment A and from data for the solvent-treated group for experiment B. NS, nonsignificant.

In previous experiments when drug administration was started at the day of inoculation, the observed delay in PrPres accumulation was still visible long after cessation of treatment. To explore this phenomenon, mice were sacrificed at 150 dpi, 30 days after the treatment was stopped. In the groups treated with 2.5 mg of AmB and 25 mg of MS-8209 per kg, PrPres accumulation was significantly lower than in the 2.5-mg/kg MS-8209- or solvent-treated groups (Fig. 1). Thus, the drug effect on PrPres could persist at least 30 days after the end of the drug therapy.

PrPres accumulation is thought to induce astrocyte activation and therefore the upregulation of GFAP. At 120 dpi, GFAP gene expression was lower in treated groups than in controls and appeared to correlate with the reduction of PrPres accumulation (Fig. 2). After 1 month without treatment (150 dpi), levels of GFAP gene expression were similar among all groups. Thus, even after polyene antibiotic administration, PrPres accumulation and GFAP gene expression seemed to fluctuate together.

Effect of long-term drug administration beginning in the late stage of the disease. It has been shown that the efficiency of treatment initiated at the day of inoculation is time and dose dependent in hamsters (1, 32). Because of the promising results regarding increased survival time after a 1-month treat-

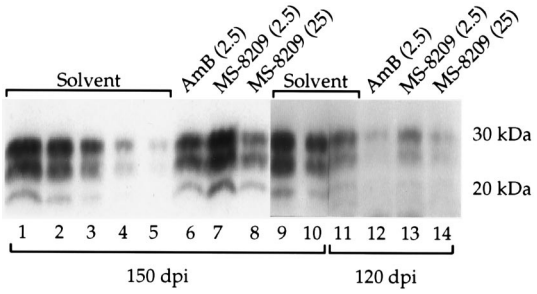

FIG. 1. Samples represent a pool of three mouse brains. Lanes: 1 to 5, twofold dilutions of the solvent-treated group at 150 dpi; 6 to 8, groups treated as indicated at 150 dpi; 9 and 10, 1/8 and 1/16 dilutions of the solvent-treated group at 150 dpi; 11, the solvent-treated group at 120 dpi; 12 to 14, drug-treated groups at 120 dpi. Samples in lanes 1 to 8 were exposed for 1 min; those in lanes 9 to 14 were exposed for 5 min. Neither AmB nor MS-8209 affected the activity of PK on PrPres digestion at a concentration 10 times higher than those in brain after a treatment at 2.5 mg/kg (data not shown). The only difference observed was a slightly enhanced immunoreactivity of PrPres when either AmB or MS-8209 was mixed with the homogenate (data not shown). Thus, the reduced level of PrPres after treatment with AmB or MS-8209 was not an artifact of PK digestion.

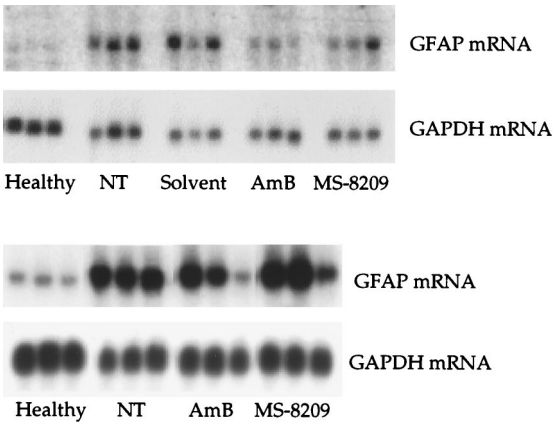

FIG. 2. Mice were not treated (NT) or treated with 2.5 mg of AmB or MS-8209 per kg; 5 μg of total RNA was loaded in each lane. (Top) Mice sacrificed at 120 dpi (at the end of treatment); (bottom) mice sacrificed at 150 dpi, 30 days after cessation of treatment.

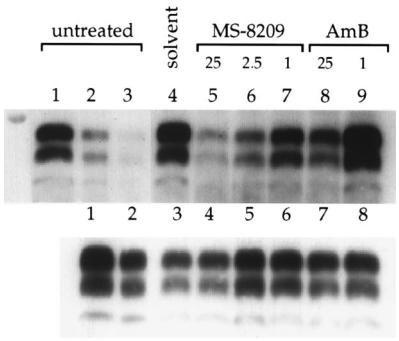

FIG. 3. Mice were sacrificed 30 days after cessation of treatment (110 or 140 dpi). Each lane represents 500 μg of a pool of three brains from the same group. (Top) Lanes: 1 to 3, twofold dilutions of untreated samples; 4, solvent treated; 5 to 9, drug treated as indicated. (Bottom) Lanes: 1 and 2, twofold dilutions of untreated samples; 3, solvent treated; 4 to 8, drug treated as indicated.

ment, in the next experiment we extended the drug administration for the entire life span, using several doses of AmB and MS-8209 beginning at 80, 110, and 140 dpi. Three animals were sacrificed 30 days after the beginning of each treatment for PrPres detection and infectivity titer evaluation, and the remaining mice were monitored for survival as usual.

Untreated and solvent-treated mice developed clinical signs at around the same time (Table 2). All of the treated groups had a prolonged incubation time when treatment was started at 80 or 110 dpi (Table 2). For treatment starting at 140 dpi, only the group treated with 25 mg of MS-8209 per kg had a significantly increased survival time (Table 2). For MS-8209, higher and longer dosages correlated with longer increases of survival time.

For the treatment beginning at 80 dpi, PrPres levels in the brain after 1 month of administration were similar among untreated, solvent-treated, and AmB-treated groups at 1 mg/kg. For the other groups, PrPres levels were decreased in inverse proportion to the dose of drug administered (Fig. 3). All of the treated groups except the group treated with AmB at 1 mg/kg showed reduced expression of the GFAP gene compared with controls (Fig. 4). These results were in accordance with those obtained with a treatment from 90 to 120 dpi at a 2.5-mg/kg dose. In contrast with the decrease in PrPres accumulation, GFAP gene expression was similar at all MS-8209 doses used. For the treatment beginning at 110 dpi, all treated groups had a PrPres level approximately half of that of the untreated group (Fig. 3), and all the groups had similar levels of GFAP gene expression (Fig. 4). No evaluation of PrPres accumulation or GFAP gene expression was done in groups treated at 140 dpi, since most of the animals died during the 30 days following the beginning of the treatment.

We evaluated infectivity titers by reinoculating brains from treated mice. Comparison of the incubation time with the titration curve obtained for the original inoculum gave an approximate infectious titer. Survival times of recipient mice inoculated with brain homogenate of mice treated from day 80 postinoculation (p.i.) were significantly different from those of the untreated groups, with a calculated titer 100-fold lower (Table 3). Survival times of recipient mice inoculated with brain homogenate of mice treated from day 110 p.i. were not significantly different from those for the untreated group (Table 3). Thus, polyene antibiotics appeared to reduce the prop-

TABLE 2. Survival times of treated and untreated mice after treatments beginning at 80, 110, or 140 dpi and continued until death with AmB or MS-8209[a]

Drug	80 dpi		110 dpi		140 dpi	
	Survival time	Delay	Survival time	Delay	Survival time	Delay
None			158.5 ± 2.6 (15)			
Solvent (NMG)	158 ± 3.1 (6)	0.5^{NS}	160 ± 2.5 (6)	1.5^{NS}	157 ± 1.2 (6)	-1.5^{NS}
AmB						
1 mg/kg	187 ± 1.4 (8)	29**	182 ± 1.4 (8)	22**	164 ± 2.2 (12)	$7^{\$}$
2.5 mg/kg	196 ± 2.3 (8)	38**	182 ± 1.6 (8)	22**	167 ± 2.5 (11)	$10^{\$}$
MS-8209						
1 mg/kg	187 ± 2.4 (8)	29**	173 ± 3.1 (8)	13**	164 ± 2.1 (10)	7^{NS}
2.5 mg/kg	198 ± 1.3 (8)	40**	180 ± 1.3 (8)	20**	165 ± 2.2 (11)	$8^{\$}$
25 mg/kg	221 ± 4.1 (8)	63**	190 ± 2.2 (8)	30**	172 ± 3.4 (12)	15*

[a] Survival times are expressed in days ± standard error followed by the effective in parentheses. The untreated group was the same for all the experiments. Delays, expressed in days, were calculated from data for the solvent-treated group. Significantly different from the untreated and solvent treated group: **, $P < 0.01$; *, $P < 0.05$. Significantly different from the untreated group: $, $P < 0.05$. NS, nonsignificant (Mann-Whitney test). During treatment beginning at 140 dpi, acute toxicity was observed in treated animals for the first 2 days of manipulation: solvent (4 of 12), MS-8209 at 2.5 (1 of 12) or (2 of 12) 1 mg/kg, and AmB at 2.5 or 1 mg/kg (1 of 12). Death occurred 10 to 20 s after injection. All animals presenting these symptoms were excluded from statistical analysis.

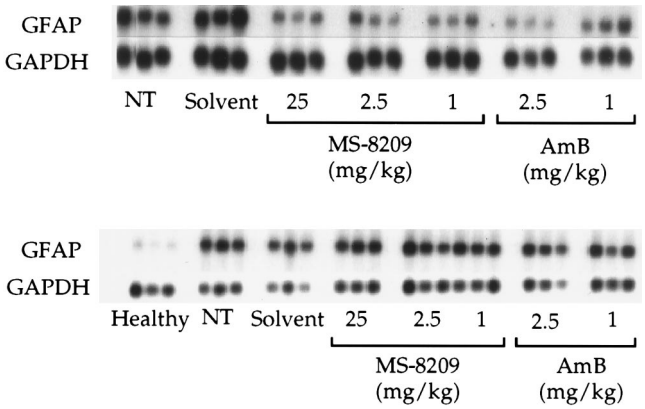

FIG. 4. Administration began either at 80 dpi (top) or 110 dpi (bottom). Mice were not treated (NT) or treated with different doses of AmB or MS-8209 as indicated. Mice were sacrificed 30 days later (110 or 140 dpi); 5 μg of total RNA was loaded in each lane.

agation of the TSE agent in the brain after the onset of the neuroinvasion.

DISCUSSION

The aim of this study was to determine if polyene antibiotics administered during the later stages of murine scrapie could have an effect on the different parameters of the experimental infection. Because the increase in infectivity and PrPres accumulation in the brain starts to be detectable around 60 to 80 dpi in our scrapie model (C57BL/6 mice; C506M3 scrapie strain), we treated animals after 80 to 90 dpi. Polyene antibiotics increased survival time when administration was initiated very late in the disease. PrPres accumulation, GFAP gene expression, and infectious titer were reduced in the brain following drug administration if treatment was initiated before the appearance of neuropathological injuries. Furthermore, since it is known that there are high agent titers in the brain prior to the onset of the treatment used in this study, these data demonstrate that polyene antibiotics are effective inhibitors of TSE pathogenesis in brain tissue itself.

This study examines the use of polyene antibiotics in treatment of experimental model of scrapie. Polyene antibiotics are efficient in both i.c.- and i.p.-infected animals (15, 31). As shown in this report, they are the only drugs effective after late administration. However, their effectiveness is restricted to certain scrapie strains (2, 11). The mechanism of this restriction is not yet understood. The other class of antiscrapie drugs, the polyanions, behave differently from AmB. Polyanions are effective only when given a short period of time after infection. Furthermore, most of the polyanions are not efficient in i.c.-infected animals (16, 19). Only HPA-23 and Congo red are slightly effective on both i.c.- and i.p.-infected animals (17, 20).

As suggested previously by Pocchiari et al. (32), AmB might act by blocking the primary infection process rather than directly on the inoculum (11). In this case, the increased incubation time would be due to a reduction of effective TSE agent load (23). Consequently, for a given day p.i., PrPres levels would be reduced, as observed with early treatment (1, 2). In this study, we initiated treatment of the animals at a time when the course of the disease was independent of TSE agent multiplication in peripheral organs (22, 30). Similar to findings for early treatment, we observed here both an increase in survival time and a delay in PrPres accumulation. Furthermore, in our experience and that of others, polyene antibiotics decrease the apparent infectious titer of the scrapie agent in the brain (26, 32).

Delays in survival time are correlated with the length of the drug treatment regardless of time of initial administration. However, late treatment seems to be as efficient as or even better than early treatment. For instance, increased survival obtained after a treatment from 90 to 120 dpi (1-month treatment) ranges from 6 to 10% of the total incubation time, which is comparable to 2 months of treatment beginning at the inoculation day (2). Some accessory mechanisms in addition to an effect on TSE agent propagation might be involved in the effectiveness of late treatment. A neuroprotective effect by AmB could be excluded, as AmB possesses neurotoxic activity in vivo (37). On the other hand, AmB could not have an effect on PrPres-mediated toxicity (8, 27), as it possesses an immunostimulatory activity on peripheral mononuclear cells (35, 38) and is by itself a prooxidant (6). Finally, polyene antibiotics may reduce the process of PrPres accumulation which is linked to TSE agent replication. The experiments of Caughey and Raymond contradict this hypothesis because they have shown that AmB, unlike polyanions, does not prevent PrPres generation in scrapie-infected cells (13).

Thus, we might assume that polyene antibiotics act directly on the spread of the TSE agent at the brain level, perhaps by disrupting interaction between the TSE agent and the normal cellular PrP molecules (7, 10).

At a molecular level, some characteristics of polyene antibiotics underline their possible close interaction with the infectious process. AmB and its derivatives affect mammalian cells by interacting strongly with membrane sterols (4). Membrane cholesterol-rich domains, known as rafts, have recently been implicated in PrPres generation (34). Modification of membrane properties (36) or of the raft characteristics by polyene antibiotics could have an effect on TSE pathogenesis.

The effects of AmB and MS-8209 are similar with respect to incubation time. MS-8209 differs from AmB by the graft of a hydrophilic moiety (33). This finding suggested that the polyene moiety common to both drugs is important in the mechanism leading to the prolongation of survival time. AmB had generally a better effect on PrPres accumulation than did MS-8209. Therefore, it would be of great interest to test other derivatives of AmB with different hydrophilic moieties to observe the effects on PrPres accumulation. These drugs could be good tools with which to investigate the relationship between TSE agent propagation and PrPres accumulation.

As potential therapeutic drugs, the polyene antibiotics are the only ones to have an effect after late administration. We

TABLE 3. Incubation times of recipient mice inoculated with brain homogenates of mice treated from days 80 or 110 p.i.[a]

Inoculum	Period of treatment (dpi)	Incubation time (days ± SEM); $n = 6$	Difference in titer (log LD_{50}/g of brain)
None	80–110	164.8 ± 4.4	
AmB	80–110	176.8 ± 3.8	−2
MS-8209	80–110	178.4 ± 3.6	−2.1
None	110–140	157.2 ± 4.1	
AmB	110–140	164.7 ± 3	−1.3
MS-8209	110–140	166.2 ± 3.7	−1

[a] Three animals were sacrificed 30 days after the beginning of the treatment. Homogenates were pooled in equal parts and injected i.c. into recipient mice as described in Materials and Methods. Brains used for inoculum were from animals treated with 2.5 mg of both drugs per kg from the previous experiment.

have to point out that the effect of a 2.5-mg/kg treatment from 80 dpi was similar to that obtained with long-term treatment at the same dose beginning at the day of inoculation (15). Indeed, for MS-8209 and AmB, we obtained 26 and 30% increases in survival time, respectively, with long-term treatment (15), compared with 24 and 25% for treatment from 80 dpi (at a dose of 2.5 mg/kg). The only reported treatment of Creuzfeldt-Jakob disease patients with AmB (25) was not effective. However, as emphasized by the present results, patients in the clinical period of the disease may require a higher dose of polyene antibiotic. Furthermore, as the effect of polyene antibiotics seems to be restricted to certain scrapie strains (2), prolongation of survival time might be observed only in certain cases of Creuzfeldt-Jakob disease due to the possible variability of agent strains causing this disease. Nevertheless, it remains of great interest to test these drugs in various situations of natural disease in humans or in domestic animals.

In summary, polyene antibiotics are the only class of antiscrapie drugs which induce a prolongation of survival time when administered in the later stages of the scrapie disease. In some cases, the prolongation of survival time was as extensive as with early treatment.

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