

Inhibiting scrapie neuroinvasion by polyene antibiotic treatment of SCID mice

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The polyene antibiotic MS-8209 is currently one of the most effective drugs in the treatment of experimental scrapie. However, its mechanism of action and its site of intervention in the pathogenetical process of scrapie infection are largely unknown. It has been shown previously that the infection of immunodeficient SCID mice by the peripheral route provides a reliable model for direct scrapie neuroinvasion, bypassing the lymphoreticular system. Indeed, a proportion of SCID mice develop scrapie after a similar time to immunocompetent mice, despite their severe immune impairment. This model is now used to clarify the targets of MS-8209. In SCID mice, MS-8209 treatment protected against infection but did not prolong survival time. In SCID mice immunologically reconstituted prior to inoculation, the drug delayed the disease without an effect on the attack rate. These findings strongly suggest that MS-8209 acts by hampering the first step of the neuroinvasion process, i.e. the uptake of the infectious agent by peripheral nerve endings. The mechanism leading to the inhibition of agent propagation to nervous cells is discussed with regard to the properties of polyene antibiotics.

Introduction

Scrapie is a neurodegenerative disease that naturally affects sheep and goats. It belongs to the group of transmissible spongiform encephalopathies (TSE) that also includes bovine spongiform encephalopathy in cattle and Creutzfeldt–Jakob disease in humans. A main pathological hallmark of TSE is the accumulation of a partially protease-resistant isoform (PrPres) of the host-encoded prion protein (PrP^C), mainly in the central nervous system (Bolton *et al.*, 1982; Prusiner, 1982). The lymphoreticular system (LRS) plays an initial role in scrapie pathogenesis, both in the natural disease and in animals experimentally infected by the peripheral route (Kimberlin & Walker, 1988; Mabbott *et al.*, 1998); indeed, organs of the LRS and particularly the spleen are infectious (Kimberlin & Walker, 1979) and exhibit PrPres accumulation long before neuroinvasion occurs (Grathwohl *et al.*, 1996; van Keulen *et al.*, 1996). Within these organs, follicular dendritic cells would

constitute the best candidate for supporting TSE agent replication (Fraser & Farquhar, 1987; McBride *et al.*, 1992; Muramoto *et al.*, 1993), while more recently a key role of B lymphocytes has been proposed (Klein *et al.*, 1997).

Experiments based on the reconstitution of PrP^{0/0} mice with PrP^{+/+} cells in the LRS and PrP^{+/+} neurografts have shown that a tissue compartment expressing PrP is necessary for the transfer of scrapie infectivity from the sites of peripheral replication to the central nervous system (Blättler *et al.*, 1997). Studies of the infection pathway point towards the peripheral nervous system (PNS) as a link between the LRS and the thoracic spinal cord (Kimberlin & Walker, 1988). Moreover, results of splenectomy experiments have suggested that a direct neuroinvasion can occur via nerve endings in the peritoneal wall, bypassing the first replication step in the spleen (Kimberlin & Walker, 1986). We previously studied the role of the immune system in severe combined immunodeficiency (SCID) mice (Lasmézas *et al.*, 1996*a*), as these mice lack functional lymphocytes and follicular dendritic cells (Bosma & Carroll, 1991). Despite their severe LRS impairment, a proportion of these mice were infectable by the intra-

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Table 1. Effect of MS-8209 on scrapie infection of SCID and R-SCID mice

Eighty-one SCID mice and 37 R-SCID mice were initially infected with scrapie. Ten SCID mice and seven R-SCID mice died of intercurrent diseases before the first case of scrapie and were negative for brain PrPres accumulation. They were excluded from the total number of mice. Two MS-8209-treated R-SCID mice were also excluded; they were killed intercurrently at 246 and 287 days p.i. but had a weak PrPres accumulation in their brains. One NMG-treated R-SCID mouse was excluded: it died from scrapie at 469 days p.i. but had more than 2-fold higher Ig levels compared with CB17 mice, suggesting a leaky phenotype. Mice were considered positive for scrapie on the basis of clinical signs and on Western blot detection of PrPres in brains. ns, Not significant. Immunological status and survival time are shown as means \pm SEM.

Mice	Treatment	Diseased mice/total	Transmission (%)	Immunological status (%)*	Survival time (days)	Delay (days)
SCID	MS-8209	5/35	14†	5 \pm 1	312 \pm 10	} NS
	NMG	16/36	44	7 \pm 1	320 \pm 7	
R-SCID	MS-8209	10/12	83	102 \pm 3	378 \pm 7	} + 34‡
	NMG	14/15	93	105 \pm 6	344 \pm 9	
CB17	NMG	21/21	100	100	327 \pm 4	} + 17‡

* Immunological status was assessed by serum Ig quantification. Values are expressed as percentages of CB17 controls.

† Statistically significant: $P < 0.01$ (*t*-test).

‡ Statistically significant: $P < 0.05$ (Mann–Whitney test).

peritoneal route with the C506M3 scrapie strain and had an incubation time similar to immunocompetent mice, demonstrating the occurrence of a direct neuroinvasion (Lasmézas *et al.*, 1996a). SCID mice therefore represent a unique model for the specific study of the spread of the infectious agent into the PNS. This constitutes a critical step in scrapie propagation, as illustrated in IM mice infected intraperitoneally with the 87V scrapie strain, in which neuroinvasion seems not to occur although infectivity has reached a plateau in the spleen (Collis & Kimberlin, 1985). Theoretically, this step would represent an ideal target for therapeutic intervention, as it would hinder propagation of the infectious agent to the replication sites that are associated with neurodegeneration.

Of the drugs showing an efficiency against experimental TSE, only one category seems amenable to the attainment of this goal. Polyene antibiotics such as amphotericin B (AmB) and the less-toxic derivative MS-8209 delay clinical disease, even when administered at a late stage of infection, with an efficiency similar to that of long-term treatment starting on the day of inoculation (Demaimay *et al.*, 1997), as opposed to polyanions, for example, the effect of which is limited to the primary replication phase in the LRS (Farquhar & Dickinson, 1986).

The aim of this study was to define the targets of MS-8209 and to investigate whether the PNS would be a suitable site of intervention to decrease the incidence of effective infection after peripheral contamination with TSE agents. We therefore studied the effects of MS-8209 in our model of SCID mice infected intraperitoneally with the scrapie agent. Immunologically reconstituted SCID (R-SCID) mice served as controls.

Methods

■ Scrapie infection and MS-8209 treatment of SCID mice.

Seven- to nine-week-old SCID mice were inoculated intraperitoneally with 100 μ l 0.2% (w/v) scrapie brain homogenate from terminal C506M3 scrapie-infected C57BL/6 mice (Lasmézas *et al.*, 1996b). They were then treated immediately with MS-8209 (Mayoly Spindler Laboratories, Chatou, France) by the intraperitoneal route at 25 mg/kg body weight, each day for 2 weeks. A control group was treated with the MS-8209 solvent, *N*-methylglucamine (NMG; Sigma).

■ Immune reconstitution and scrapie infection of SCID mice.

For immune reconstitution, 4–5-week-old SCID mice were injected intraperitoneally with a spleen cell suspension obtained from pooling the spleens of mice from the parental mouse strain CB17 (from which SCID mice were derived by spontaneous mutation). The equivalent of one CB17 mouse spleen was used to reconstitute two SCID mice. Five weeks later, serum immunoglobulin (Ig) levels of six R-SCID mice were quantified and compared with those of CB17 mice. Sera were serially diluted in PBS plus 0.005% BSA. Serum samples were then loaded onto a nitrocellulose membrane (Schleicher & Schuell) by using a slot blot apparatus (Bio-Rad). Mouse Ig was detected with a peroxidase-conjugated antibody (Southern Biotechnology Associates). Immunodetection was carried out with an enhanced chemiluminescence kit (Amersham) and signals were quantified by using the NIH-Image program for autoradiographic films (Wayne Rasband, National Institute of Health, USA). R-SCID mice were then intraperitoneally inoculated with 100 μ l 0.2% (w/v) brain homogenate and treated as SCID mice. One group of CB17 mice was similarly inoculated and treated with MS-8209 solvent for 1 week to serve as a further standard of infection in immunocompetent mice harbouring the same genetic background as SCID mice.

The incubation period of scrapie-infected animals is regarded as the time between inoculation and death. All mice were sacrificed at the

terminal stage of the disease. At this point, immunological status was verified for all the mice (including the survivors). Spleens and brains were kept for PrPres analysis and serum for Ig measurements.

■ **PrPres analysis.** Brains (one hemisphere) and spleens were homogenized at 20% (w/v) in a sterile 5% glucose solution with a Ribolyser (Hybaid). PrPres was purified according to a previously reported protocol for isolation of scrapie-associated fibrils (Lasmézas *et al.*, 1996b). Proteinase K was used at 10 µg/ml. Samples were loaded on a 12% polyacrylamide gel and then transferred onto a nitrocellulose membrane (Schleicher & Schuell). Mouse PrPres was visualized by immunoblot with the polyclonal anti-PrP antibody JB007 (Demaimay *et al.*, 1997) diluted 1:5000. Immunoreactivity was detected with an enhanced chemiluminescence kit (Amersham) and visualized by autoradiography.

Results

Immunological status of SCID and R-SCID mice

Ig measurements conducted at the time of reconstitution showed the efficiency of the immune restoration in R-SCID mice (data not shown). At the terminal stage of the disease, all R-SCID mice still exhibited Ig levels similar to CB17 control mice (Table 1). No immunological differences were observed between diseased and survivor SCID mice and no differences were observed between MS-8209- and solvent-treated groups at the terminal stage of the disease: similar, very low levels of serum Ig were present in these mice (Table 1).

Treatment of SCID mice with MS-8209

The first scrapie cases in SCID mice, as assessed by clinical signs and PrPres detection in the brain, appeared at 282 days post-inoculation (p.i.) in the solvent-treated group and at 286 days p.i. in the MS-8209-treated group (Fig. 1). Forty-four per cent of solvent-treated SCID mice developed scrapie, with a mean incubation period of 320 days p.i., similar to that of mice from the parental strain CB17 (Table 1). Only 14% of SCID mice developed the disease when treated with MS-8209, with

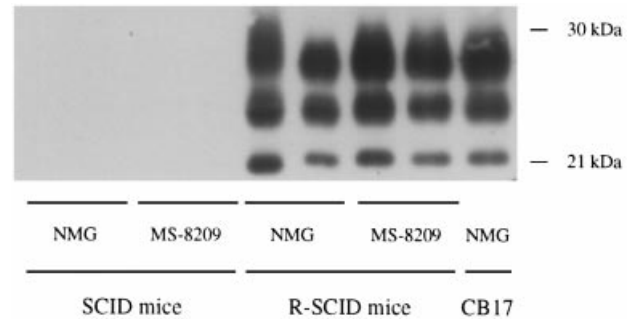


Fig. 2. PrPres detection in the spleens of SCID, R-SCID and CB17 mice at the terminal stage of the disease. Mice were treated with MS-8209 (25 mg/kg) or with its solvent (NMG).

a mean incubation period (312 days p.i.) similar to that of solvent-treated SCID mice (Table 1). At the terminal stage of the disease, no PrPres accumulation could be detected in the spleens of either solvent- or MS-8209-treated SCID mice (Fig. 2).

Treatment of R-SCID mice with MS-8209

Almost 100% of scrapie-infected, solvent-treated R-SCID mice developed the disease (Table 1), albeit with a significant delay compared with the SCID group and the CB17 group (incubation period 344 days p.i.; $P < 0.05$, Mann–Whitney test). It is noteworthy that one R-SCID mouse from the solvent-treated group and two from the MS-8209-treated group were still alive at 500 days p.i.

In contrast to the effects observed in SCID mice, MS-8209 treatment did not increase the number of R-SCID survivors but prolonged the incubation period by 34 days (Table 1; $P < 0.05$, Mann–Whitney test). The first positive cases appeared at 280 days p.i. in the solvent-treated group and at 344 days p.i. in the MS-8209-treated group (Fig. 1). PrPres was detected in the spleens of all terminally ill R-SCID and CB17 mice, with no

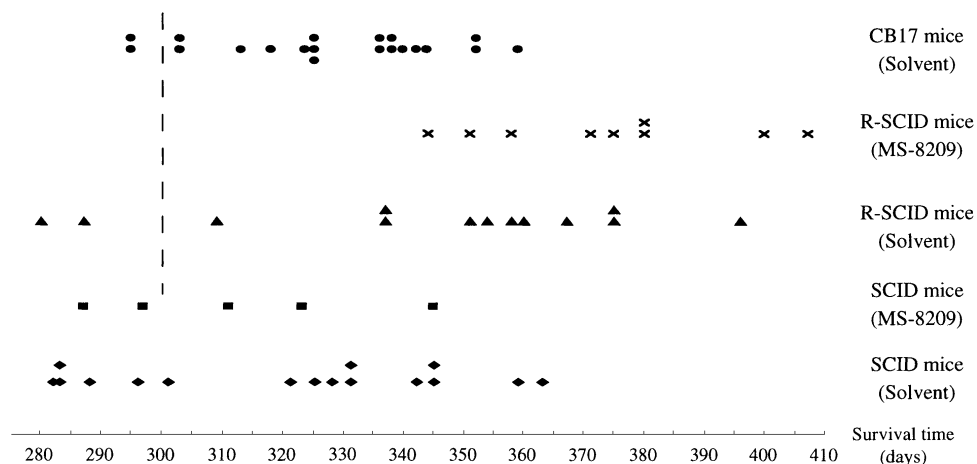


Fig. 1. Individual survival times of scrapie-infected SCID, R-SCID and CB17 mice, treated with MS-8209 or with its solvent. 'Early' cases in R-SCID mice are defined as those occurring before 300 days p.i. (to the left of the dotted line).

differences between solvent- and MS-8209-treated animals (Fig. 2).

Discussion

Immunodeficient SCID mice have been used successfully to define the involvement of the immune system in TSE pathogenesis (Fraser *et al.*, 1996; Lasmézas *et al.*, 1996a; O'Rourke *et al.*, 1994). Our study confirms earlier results obtained with the C506M3 scrapie strain (Lasmézas *et al.*, 1996a): only a proportion of SCID mice (44%) were infected; the incubation period was similar to normal CB17 mice but there was no detectable PrPres in the spleen (Table 1 and Fig. 2). This reflects that (i) in those mice that were infected, neuroinvasion occurred primarily by bypassing the defective LRS and (ii) there is a clearance of the inoculated agent as a consequence of the absence of its replication in the LRS; thus the transmission rate depends directly on the probability of the agent being taken up by the PNS. Immune reconstitution of SCID mice with splenocytes restored scrapie susceptibility fully, but the incubation period was prolonged compared with that of CB17 and SCID mice. This suggests that, even if the immune restoration is sufficient to allow infection of all the mice, the number of LRS replication sites for the agent is still smaller than in normal mice, resulting in delayed neuroinvasion. Indeed, lymphoid reconstitution can still be incomplete, despite the fact that Ig levels are normal in R-SCID mice 5 weeks after immune reconstitution (Riggs & Stowers, 1996). Moreover, when individual incubation times are compared (Fig. 1), it is noteworthy that short incubation times ('early' cases, defined as those occurring before 300 days p.i.), which were obviously mainly a feature of SCID mice, were also observed in two R-SCID mice, suggesting the occurrence of direct entry via peripheral nerves. In fact, R-SCID mice seem to constitute an intermediate between SCID mice, where all infections occur via direct neuroinvasion (this study; Lasmézas *et al.*, 1996a), and immunologically normal mice, where the infectious agent is first trapped and replicates in the LRS before the neuroinvasion phase occurs, whatever the incubation period.

A 2 week MS-8209 treatment of SCID mice, performed immediately after scrapie infection, reduced the transmission rate by 3-fold when compared with the solvent controls (Table 1). As these two groups of SCID mice were similarly immunodeficient, the difference observed was due specifically to the administration of MS-8209. The fact that MS-8209 was efficient in SCID mice indicates that, in this case, the effects of the drug were independent of a functional LRS. The fact that MS-8209 prevented scrapie infection in SCID mice without delaying clinical signs of scrapie, an on/off effect, shows that the drug interfered with the early steps of the neuroinvasion process.

In other words, these results indicate that MS-8209 prevents the propagation of the scrapie agent to the PNS. This

blockage leads to the clearance of the agent because of the absence of sustained replication in the LRS compartments, and subsequently to the lack of infection. In immunocompetent R-SCID mice, the LRS acts as a peripheral reservoir of infectivity; thus the hindrance of nervous uptake achieved by MS-8209, which is lifted at the end of the 2 week treatment, cannot prevent infection and leads to delayed neuroinvasion (a 10% increase in survival time). As another consequence, no more 'early' cases, most likely related to direct neuroinvasion, were observed in MS-8209-treated R-SCID mice (Fig. 1).

Considering the properties of polyene antibiotics and SCID mice, two mechanisms of action could account for the inhibition of scrapie agent propagation to nervous cells. Firstly, the drug could directly disrupt the fine terminal processes of the nerves linking the spleen, lymph nodes and peritoneal wall to the thoracic spinal cord. Indeed, the chemical structure of this AmB derivative enables it to interact with cell membrane components, resulting in the alteration of plasma membrane properties (Vertut-Doi *et al.*, 1994) and subsequently of the uptake of the scrapie agent. Secondly, MS-8209 may reduce the amount of the agent present in the vicinity of nerve endings by increasing the phagocytic clearance of the inoculum. Indeed, macrophages are able to sequester (Manuelidis *et al.*, 1997) and to a certain extent destroy (Carp & Callahan, 1982) infectivity. Furthermore, macrophages are functional in SCID mice (Bosma & Carroll, 1991) and MS-8209, like AmB, is known to activate macrophage functions *in vitro* (P. Clayette, personal communication; Wolf & Massof, 1990).

Overall, this study identifies the neuroimmune interface, in its full definition as the junction between the LRS and the PNS, i.e. the site of scrapie neuroinvasion, as (i) a weak link in scrapie pathogenesis, whether or not the immune system is functional, and (ii) a target for anti-TSE drugs, particularly in case of peripheral TSE contamination. This opens new insights into therapeutic strategies, as this study provides evidence that a treatment combining a drug targeted to this compartment, such as MS-8209, together with a drug interfering with the replication of TSE agents in the LRS would offer the best chances of success.

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