

Infectivity in extraneural tissues following intraocular scrapie infection

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Intraocular (i.o.) infection of mice with scrapie produces strain-specific targeting of replication and subsequent pathology within the visual system projection areas in the CNS, but also initiates an extraneural infection. Following i.o. infection with ME7 scrapie, infectivity was detected 24 h later in the Harderian gland, the superficial cervical lymph nodes (SCLNs) and the spleen, but not until 20 days in Peyer's patches and inguinal lymph nodes (ILNs). Persistent low levels of infectivity were found in the Harderian gland (which lies within the orbit), but the presence of PrP could not be confirmed by immunolabelling or Western blotting. SCLNs contained maximal amounts of infectivity by 20 days post-infection and remained at this level throughout the incubation period. ILNs reached a similar plateau at

60 days, as did Peyer's patches at 80 days and spleen at 100 days. Further investigation of the role of the spleen in pathogenesis showed that in contrast to ME7 scrapie, mice infected with 79A scrapie had high levels of infectivity in the spleen by 20 days post-infection, irrespective of the route of infection. In addition, the disease developed more rapidly following direct intrasplenic infection with ME7 scrapie than with intraperitoneal infection. Splenectomy at 7 days either before or after i.o. infection had no effect on the incubation period. These results indicate that the rate of replication of infectivity is both tissue and scrapie-strain dependent, and that extraneural spread of infection can occur via the lymphatic system.

Introduction

Extraneural routes of infection with scrapie have been used to establish the sequence of events preceding infection of the CNS (Scott, 1993). It was originally demonstrated by Eklund *et al.* (1967) that following subcutaneous infection of mice with the 'Chandler' scrapie isolate, infection is initially detected in the spleen and subsequently in lymph nodes, thymus and salivary glands before spreading to the spinal cord and brain. Many other studies, notably by Kimberlin & Walker (see review 1988*a*) have substantiated these results using several strains of scrapie, and the importance of the spleen as a site of replication has been shown by the prolongation of incubation period resulting from infection of splenectomized (Fraser & Dickinson, 1970; Kimberlin & Walker, 1989*a*) or genetically asplenic mice (Dickinson & Fraser, 1972). The disease-specific form of PrP, a membrane glycoprotein, can be detected in spleen early in the incubation period of some scrapie strains using immunoblotting techniques (Doi *et al.*, 1988; Race &

Ernst, 1992; Farquhar *et al.*, 1994). The accumulation of the abnormal form of PrP is idiopathic for the spongiform encephalopathies, and this protein appears to play a key role in these diseases (Hope & Chong, 1994).

The intraocular (i.o.) route of infection has been used to target scrapie infectivity and subsequent pathology to the neuroanatomical projections of the retina in mouse (Fraser, 1982; Fraser & Dickinson, 1985; Scott *et al.*, 1992) and hamster (Buyukmihci *et al.*, 1983; Kimberlin & Walker, 1986). The progression of the infection is consistent with the anterograde spread of scrapie within the optic nerve, but there is also independent evidence for the parallel development of extraneural infection. This evidence comes from three sources: the first is an experiment designed to establish the minimum period necessary to establish disease in the CNS following i.o. infection. Mice enucleated up to 14 days after i.o. infection develop scrapie after a prolonged incubation period, typical of extraneural infection, and also lack the asymmetrical lesions which indicate direct infection via the optic nerve (Scott & Fraser, 1989). Secondly, a similar prolongation of incubation period has been found following i.o. infection of mice treated with monosodium glutamate (MSG) as neonates (Foster *et al.*,

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1990). This treatment specifically destroys the retinal ganglion cells thus preventing the spread of infection through the optic nerve. Lastly, disease develops after a prolonged incubation period in a proportion of mice infected merely by instilling inoculum on to the conjunctiva (Scott *et al.*, 1993). However, the route of spread of extraneural infection is unknown.

In this study, extraneural pathogenesis following i.o. infection with ME7 scrapie was investigated through sequential assay of infectivity in lymphoreticular tissues. The role of the spleen was studied by comparing ME7 and 79A strains of scrapie, and also the effect on incubation period of direct intrasplenic infection and splenectomy either before or after i.o. infection.

Methods

Five inbred mouse strains (C57BL/FaBtDk, C3H/LaDk, SM/RrChBtDk, VL/Dk and BSC/Dk) were used in these experiments, some of which were conducted as part of another series, hence the diversity of strains. All strains carry the s7 allele of the *Smc* gene (Bruce *et al.*, 1991). Groups of mice were infected with either ME7 or 79A strains of scrapie, both of which have been biologically cloned by at least three intracerebral (i.c.) passages at high dilution (described by Bruce, 1996). Inocula were prepared as 10% homogenates in saline of brain tissue from terminally affected mice. Infection was introduced by one of five routes: i.c. (into right parietal cortex); i.o. (bilaterally into the vitreous chamber, except in the splenectomy experiment where infection was unilateral); intraperitoneal (i.p.), intravenous (i.v.) (into tail vein); and intrasplenic (using a 27 gauge needle inserted about 4 mm along the length of the spleen). The volume of inoculum injected was 20 μ l for i.c. tissue assays. All other injections were of 1 μ l, including i.c. injected controls on the titre of the inoculum, with the exception of the bilateral i.o. infection (1 μ l per eye, and 2 μ l for i.c. control mice). Splenectomy, i.o. and intrasplenic injections were performed under barbiturate anaesthesia. Tissues were removed for bioassay from groups of three VL/DK mice at each time-point using aseptic techniques and appropriate safeguards to prevent contamination of individual tissue pools. These were (in order of removal): Harderian gland, superficial cervical lymph nodes (SCLNs), inguinal lymph nodes (ILNs) and Peyer's patches. Five Peyer's patches were dissected from the gut wall of each mouse. Tissues were taken at 24 h, 10 days, 20 days and at 20 day intervals up to 160 days after i.o. infection. Assays later than 160 days were not considered necessary to determine the sequence of initiation of replication. Spleen assays were from a replicate experiment with tissues taken at 24 h, and 20, 60, 100 and 140 days after i.o. infection. The incubation period for the donor mouse group following i.o. injection was 226 ± 3 (mean \pm SE). All tissues were frozen at -20°C until assay mice were available. Infectivity levels were estimated by incubation period assay: individual tissue pools, each from three mice, were prepared as 10% homogenates in saline. Groups of 8–12 assay mice were injected i.c. with 20 μ l of each tissue homogenate. All tissues except spleen were assayed in C3H/LaDk mice, spleens were assayed in BSC/Dk mice. There is no reason to expect that this would make a difference to the estimate of titre, since these strains have similar incubation periods for ME7 scrapie. Homogenates of Peyer's patches were treated with antibiotics (penicillin/streptomycin, Gibco BRL) in case of inadvertent contamination with gut contents. The mean incubation period of each group of assay mice was compared with an

appropriate dose-response curve, and the result expressed in i.c. ID₅₀ units per 20 μ l (the injected dose). It has been shown (Robinson *et al.*, 1990) that dose-response curves should be specific for the tissue being assayed; however, in these experiments, the titre in all lymphoreticular tissues was estimated using a spleen dose-response curve as dose-response curves do not exist for individual tissues. Western blots for PrP were prepared from fresh Harderian gland tissue pools taken from terminal scrapie mice and normal-brain injected age-matched controls, according to the method of Farquhar *et al.* (1994) for non-neural tissues. Conjunctival drainage to the SCLN was confirmed by infusion of Pelikan ink, which was present in the SCLNs 24 h later.

All experiments were performed under a Home Office project licence, and the numbers of mice kept to the minimum necessary to ensure a significant result.

Results and Discussion

Infectivity in extraneural tissues

The results of the sequential assay of extraneural tissues are shown in Table 1.

Harderian gland. In mice, this horseshoe-shaped gland lies within the orbit, partially encircling the optic nerve (Benirschke *et al.*, 1978). It was selected because of its large mass and proximity to the injection site. At 24 h post-infection the Harderian gland had the highest titre of the five tissues assayed, presumably due to the retention of inoculum. At 10 days infectivity was detectable but not estimable, and from 20 to 140 days the titre fluctuated between 1.6 and 2.4, rising to 3.1 at the final time-point. It is difficult to say whether the presence of infectivity in this tissue is due to replication or merely the sequestering of infection produced elsewhere.

Western blots of Harderian gland pools were prepared in an attempt to visualize PrP, but no signal was found, suggesting either a discontinuity in the relationship between infectivity and PrP similar to that shown in salivary glands of mice infected with Creutzfeldt–Jakob disease (Sakaguchi *et al.*, 1993), or that the levels were beneath the threshold that can be detected using Western blotting. The low, but consistent levels of infection suggest either replication in a small, changing population of cells, replication to a low plateau level followed by persistence, or merely a sequestering of infectivity produced elsewhere. It is interesting that Farquhar *et al.* (1994) found that in a similar mouse strain intracerebrally infected with ME7 scrapie, PrP could be sporadically demonstrated in the submaxillary gland throughout the incubation period by immunoblotting.

Superficial cervical lymph node. The SCLN is the major lymph node draining the conjunctiva (Forrester, 1987). This was confirmed in the VL/Dk mouse strain by infusion of Pelikan ink. Infectivity in SCLN increased rapidly from 2.6 at 24 h to reach a level of 4.3 units at 20 days which was then maintained as a plateau.

Table 1. Infectivity estimates from various tissue pools taken sequentially following i.o. infection of VL/Dk mice with ME7 scrapie

Data are given as mean incubation period (IP) in days \pm SE; titre is given in log i.c. ID₅₀ units/0.2 ml and *n* is the number of C3H/Dk mice which developed the disease over the number in the group. NA, Not available.

Time post-injection	Harderian gland			Superficial cervical lymph node			Inguinal lymph node			Peyer's patches			Spleen		
	IP	Titre	<i>n</i>	IP	Titre	<i>n</i>	IP	Titre	<i>n</i>	IP	Titre	<i>n</i>	IP	Titre	<i>n</i>
24 h	219 \pm 6	3.3	9/9	257 \pm 9	2.6	6/6	—	0	0/9	NA			266 \pm 1	2.4	9/9
70 days	389 \pm 53	< 1	2/9	214 \pm 5	3.4	8/8	—	0	0/9	NA			NA		
20 days	309 \pm 32	1.5	3/9	173 \pm 2	4.3	9/9	187	[4.0]	1/9	294 \pm 4	1.8	4/9	234 \pm 1	3.0	11/11
40 days	259 \pm 8	2.5	9/9	168 \pm 5	4.4	8/8	190 \pm 2	4.0	8/9	229 \pm 5	3.1	8/8	NA		
60 days	297 \pm 59	1.7	4/6	174 \pm 2	4.3	9/9	176 \pm 2	4.3	9/9	NA			225 \pm 1	3.2	8/8
80 days	264 \pm 12	2.4	9/9	173 \pm 1	4.3	8/8	172 \pm 3	4.4	9/9	181 \pm 0	4.2	9/9	NA		
100 days	NA			176 \pm 1	4.3	7/7	160 \pm 3	4.6	9/9	181 \pm 0	4.2	9/9	161 \pm 1	4.5	10/10
120 days	272 \pm 8	2.2	8/8	179 \pm 3	4.2	8/8	173 \pm 2	4.3	9/9	178 \pm 2	4.2	9/9	NA		
140 days	296 \pm 20	1.7	7/9	176 \pm 2	4.3	8/8	169 \pm 2	4.4	9/9	179 \pm 2	4.2	9/9	162 \pm 1	4.5	12/12
160 days	229 \pm 8	3.1	7/7	175 \pm 1	4.3	9/9	168 \pm 1	4.4	8/8	179 \pm 2	4.2	9/9	NA		

Inguinal lymph node. ILNs were taken to represent the lymphoreticular system distant from the site of infection. One mouse in the bioassay group of nine infected with ILN from a 1-day donor developed the disease with a relatively short incubation period of 187 days. From 60 to 160 days, titres remained between 4.3 and 4.6.

Peyer's patches. Peyer's patches were collected as an indicator of spread of infectivity via the alimentary canal. Since the conjunctiva drains to the lachrymal sac at the back of the throat, it is possible that some of the inoculum was swallowed. Infection was detected at low levels in less than half of the assay mice at 20 days, and a maximum titre of 4.2 was maintained from 80 days (although no assay was available for 60 days).

Spleen. Spleen was sampled as the major organ of the lymphoreticular system. Significant infectivity was detected at 24 h, 20 and 60 days; maximum titre was reached at 100 days.

Infectivity was detectable from 24 h in Harderian gland, SCLN and spleen, but was not detected in ILN until 20 days. Although the first two time-points were not available for Peyer's patches, the low titre at 20 days suggests that these would also have been negative. This sequence indicates that SCLN, the draining lymph node and the spleen have early access to infection which only later becomes available to Peyer's patches and ILNs. Downward spread of infection from the lumen of the alimentary canal is therefore unlikely. The sequence in which the tissues reached maximum titre (disregarding the Harderian gland) is different from the initial

detection of infectivity: SCLN, ILN, Peyer's patches and finally spleen. This could suggest either tissue-dependent differences in the rate of replication or a rapid increase in replication following a variable period of time at a lower rate. However, the data show that titres in SCLN, Peyer's patches and spleen rose steadily to a peak, indicating the former interpretation.

This sequence differs from the progression of infection following i.p. infection, where with a similar mouse model (ME7 in CW mice), Kimberlin & Walker (1988*b*) showed that titres in SCLN and spleen both rose rapidly in the first 4 weeks of the incubation period. In the present study, maximum spleen titres were not reached until 100 days. This result provoked further investigation of the role of the spleen.

The role of the spleen

The spleen titres in Table 1 came from an experiment set up in order to compare sequential infectivity in the spleen following i.o. and i.c. infection with ME7 and 79A scrapie. I.c. infection is known to initiate a peripheral pathogenesis which is presumed to result from haematogenous spread from the i.c. injection site (Millson *et al.*, 1979); however, this may depend on the volume of inoculum injected. The standard i.c. volume is 20 μ l, but in order to compare the two routes of infection, the volume was 2 μ l for both (bilateral injection of 1 μ l by i.o. route). Table 2 shows that very similar sequential titres were produced by both routes of infection; however, mice infected with 79A scrapie developed high titres by 20 days post-injection. These levels continued to rise slowly, and the final titre is over tenfold greater than that found in the ME7 infected mice. The different replication rates appear to be determined

Table 2. Comparison of infectivity estimates from sequential spleen pools taken from VL/Dk mice infected with ME7 or 79A scrapie by the i.o. or i.c. routes

Mean incubation period (IP) is given in days \pm SE, titre is in log i.c. ID₅₀ units/20 μ l; *n* is the number of BSC/Dk mice which developed scrapie over the number in the group.

Time post-injection	ME7						79A					
	Intraocular			Intracerebral			Intraocular			Intracerebral		
	IP	Titre	<i>n</i>	IP	Titre	<i>n</i>	IP	Titre	<i>n</i>	IP	Titre	<i>n</i>
24 h	266 \pm 18	2.4	9/9	230 \pm 8	3.1	12/12	194 \pm 9	1.3	8/11	259 \pm 65	0.5	2/12
20 days*	234 \pm 1	3.0	11/11	237 \pm 3	3.0	11/11	143 \pm 2	4.2	12/12	142 \pm 2	4.3	12/12
60 days	225 \pm 1	3.2	8/8	219 \pm 4	3.3	11/11	132 \pm 1	5.7	12/12	137 \pm 3	4.9	12/12
100 days	161 \pm 1	4.6	10/10	162 \pm 1	4.6	12/12	130 \pm 1	5.9	11/11	132 \pm 1	5.7	11/11
140 days	162 \pm 1	4.6	12/12	162 \pm 1	4.6	12/12						

* 23 days for 79A infected mice

Table 3. Effect of splenectomy on SM mice infected with 1 μ l of a 10% homogenate of ME7 scrapie by the i.o. route

Splenectomy	Incubation period (Mean no. of days \pm SE)	No. of mice
7 days before infection	236 \pm 6.6	15/15
7 days after infection	232 \pm 4.0	18/18
Infection alone	236 \pm 2.8	18/18

by the strain of scrapie in contrast to the different rates of replication between tissues suggested by the data in Table 1. Although the dynamics of infectivity in the spleen are not influenced by injection site in this experiment, incubation period differences do exist between various peripheral routes of infection (Kimberlin & Walker, 1989a). Since different sites of infection may restrict the access of infectivity to the spleen, the incubation period produced by direct intrasplenic infection was compared with i.p. among other routes. When C3H mice (13–18 per group) were infected with 1 μ l of a 10% homogenate of ME7 scrapie, the intrasplenic incubation period was 263 \pm 3 days, significantly shorter ($P < 0.1$) than the i.p. route (291 \pm 3), compared with the i.v. (252 \pm 6) and i.o. (232 \pm 4) routes. The i.p. route is commonly used to initiate peripheral pathogenesis which is dominated by events in the spleen (Fraser & Dickinson, 1970; Kimberlin & Walker, 1989a; Race & Ernst, 1992). The incubation periods for intrasplenically infected mice were closer to those infected by an intravenous

route which is thought to be shorter as many lymphoreticular replication sites are accessed simultaneously (Fraser, 1979; Kimberlin & Walker, 1989a).

The results given in Table 1 indicate that peripheral replication of infectivity, especially in spleen, is unlikely to contribute to the pathogenesis of i.o. infection. In order to substantiate this, the effect on the i.o. incubation period of removing the spleen, either 7 days before or 7 days after infection was examined. Table 3 shows that splenectomy had no effect on incubation period. Although infectivity was detected at an early stage in the incubation period in the Harderian glands, SCLN and spleen, these organs, innervated by the autonomic nervous system, lack the direct single neuron route to the CNS of the optic nerve. It is concluded that although peripheral pathogenesis can, in the absence of sustained i.o. infection, engender disease (i.e. after enucleation of the infected eye, or in MSG-treated mice lacking retinal ganglion cells), it is unlikely that i.o. pathogenesis is influenced by any component of this peripheral replication. Comparable situations have been found with other scrapie and scrapie-like agents. Following subcutaneous infection of goats with scrapie, infectivity was first detected in lymphatic tissue, primarily the node draining the site of inoculation (Hadlow *et al.*, 1974). Similarly, when mink are subcutaneously infected with transmissible mink encephalopathy, peripheral replication is confined to the lymph node draining the site of infection, and replication only occurs in the spleen and the rest of the lymphoreticular system after infection has been detected in the CNS (Hadlow *et al.*, 1987). Following intragastric infection with 139A scrapie, replication is initiated almost immediately in Peyer's patches; spleen titres subsequently increase, but the incubation period is unaffected by splenectomy (Kimberlin & Walker, 1989b).

The sequence of detection of infectivity in lymphoreticular tissues following i.o. infection indicates that infection is accessed initially by the draining lymph node and the spleen. Since the spleen has no afferent lymphatic connection, haematogenous spread of infection either directly or via the SCLN efferent to the venous system appears the most likely route of infection. Subsequent infection of the more remote Peyer's patches and ILN is probably also haematogenous, as the lymphoid system does not permit anterograde transport. A similar route of infection has been shown recently by Taylor *et al.* (1996), who demonstrated the efficiency of scarification as a route of infection in normal but not in SCID mice. The authors conclude that 'the absence of disease in the SCID mice indicates that infectivity does not reach the CNS directly by either the bloodstream or peripheral nerves...but that it is probably transported by the lymphatic system to the lymph nodes and spleen'. The skin has a rich superficial plexus of lymphatics and micro-organisms in peripheral lymphatics are rapidly transported to the local lymph node (Mims, 1982).

In conclusion, the results presented here confirm the importance of the draining lymph node in extraneural replication, and suggest firstly that the rate of replication of infection is both tissue and scrapie-strain specific, and secondly that the spread of extraneural infection depends on both lymphatic and haematogenous systems.

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