Journal of General Virology (1996), **77**, 1935–1940. Printed in Great Britain

Replication of scrapie in spleens of SCID mice follows reconstitution with wild-type mouse bone marrow

H. Fraser, K. L. Brown, K. Stewart, I. McConnell, P. McBride and A. Williams

Institute for Animal Health, BBSRC & MRC Neuropathogenesis Unit, West Mains Road, Edinburgh EH9 3JF, UK

SCID mice are resistant to intraperitoneal infection with 10^3 and 10^4 intracerebral ID_{50} units of ME7 scrapie agent whereas they develop disease after intracerebral challenge. However, higher doses introduced, by intraperitoneal or subcutaneous routes, produce disease. Immunocompetent mice of the same strain (CB20) developed scrapie following either intracerebral or intraperitoneal infection. Bioassay of spleens from SCID mice infected with 10^{-1} dilutions of ME7 scrapie by intraperitoneal, intracerebral or abdominal subcutaneous injection showed traces or low levels of infectivity in spleen. However, subcutaneous injection beneath the skin of the neck failed to infect the spleen. CB20 bone marrow reconstitution of SCID mice resulted in the regeneration of a normal lymphoid architecture in the spleen. Spleens from these reconstituted mice, infected intracerebrally with a 10^{-1} dilution of ME7 contained high levels of infectivity. These results suggest that the ability to replicate scrapie agent in spleen or lymphoid tissue depends on the restoration of normal lymphoid structure and in particular the presence of differentiated follicular dendritic cells. The possibility that SCID mice can select minor strains of scrapie which are normally unrecognized in cloned ME7 is discussed.

Introduction

Although scrapie is a degenerative disease only of the central nervous system (CNS) it is an infection of both central nervous and some systemic tissues, and lymphoreticular replication of infectivity can precede that in the CNS by many weeks or months. No clinical, cellular or immunological dysfunction has so far been associated with the replication of infectivity in peripheral tissues despite the high titres of agent which can occur in peripheral organs such as spleen and lymph nodes (Fraser *et al.*, 1992). There is strong evidence that the target cell permitting agent replication in the CNS is the neuron (Fraser & Dickinson, 1985) but the candidate cells which are permissive for peripheral replication have not been identified (Fraser *et al.*, 1992). There is conflicting evidence concerning a role for cells which subserve immune function in scrapie pathogenesis. Neither adult nor neonatal thymectomy has any effect on subsequent scrapie pathogenesis whereas surgical and genetic asplenia prolong the incubation period in several scrapie models (Fraser *et al.*, 1992; Fraser & Dickinson, 1970, 1978; Dickinson & Fraser, 1972). Neonatal mice are resistant to doses of peripheral infection which are infectious to adults, and treatment of adult mice with immunosuppressive doses of the corticosteroid prednisolone acetate reduces their susceptibility to that of neonates (Outram *et al.*, 1973, 1974). The host protein PrP, with a well-established association with all the scrapie-like infections, has been shown to participate in human lymphocyte activation, suggesting that it may subserve an immunological function (Cashman *et al.*, 1990). There are no agent specific antigens or immunity to any of the scrapie-like diseases, no inflammation or immunopathology, and the infection reaches the CNS from peripheral lymphoid replication sites via peripheral innervation (Fraser *et al.*, 1992; Fraser, 1979; Kimberlin & Walker, 1988). Polyanions such as dextran sulphate 500 and pentosan polysulphate, which can impede the afferent arm of immunological recognition and interfere with antigen processing and macrophage function, also prolong the scrapie incubation period and delay replication of agent in the spleen (Ehlers & Diringer, 1984; Farquhar & Dickinson, 1986).

Neither sublethal nor lethal doses of whole-body ionizing radiation, administered as single or fractionated doses, at various times before or after infection, have any effect on infectibility, incubation period, neuropathology, or on replication or levels of scrapie agent in the spleen in several scrapie models (Fraser & Farquhar, 1988; Fraser *et al.*, 1989). The major conclusion from these findings is that scrapie agent replication outside the CNS is independent of most lymphoid cells, but

Author for correspondence: Hugh Fraser.
Fax +44 131 668 3872. e-mail HughFraser@bbsrc.ac.uk

depends on non-dividing, long-lived cells which are not replaced from a stem cell population during adulthood (Fraser *et al.*, 1989). Their identity as follicular dendritic cells (FDCs) has been proposed (Fraser *et al.*, 1992; Fraser & Farquhar, 1987). The presence of cells with the morphology and location of FDCs in the spleen and lymph nodes of mice, which immunostain with anti-PrP and entrap immune complexes supports this suggestion (McBride *et al.*, 1992). However, a radiation-resistant population of lymphocytes is recognized (Miller & Cole, 1967). A new opportunity to resolve this uncertainty and to further define the role of the immune system in the peripheral pathogenesis of scrapie has arisen from the availability of the severely combined immunodeficient (SCID) mouse, which shows a resistance to peripheral infection with a murine strain of Creutzfeldt–Jakob disease (Kitamoto *et al.*, 1991). These mice have an autosomal recessive mutation that prevents the formation of functional B and T lymphocytes (Bosma *et al.*, 1983) and, in addition, possess non-functional follicular dendritic cells, a defect which can be reversed after reconstitution with isogenic bone marrow (Szakal *et al.*, 1990).

Methods

■ **ME7 infection of SCID and CB20 mice.** SCID (BALB/c, CB17 *scid/scid*) and immunocompetent CB20+/+ congenic BALB/c mice were maintained throughout the experiment in a sterile environment in a positive pressure isolator. SCID and CB20 mice were infected intracerebrally (i.c.) or intraperitoneally (i.p.) with 10^{-2} and 10^{-3} (w/v) dilutions of unspun brain homogenates from terminal ME7 scrapie-affected C57BL mice. In a second experiment, SCID mice were infected with a 10^{-1} dilution of ME7 by i.c., i.p. and two subcutaneous (s.c.) routes (into the scruff of the neck or into the skin of the ventral abdomen). Injections were carried out using a 25-gauge needle and a volume of 0·02 ml. Injection of SCID mice was carried out in an isolator using double gloves, which produced difficulty in the administration of some injections, in particular the subcutaneous abdomen injections. At the time of these injections the possibility of inadvertent i.p. injection was recorded. Following infection the animals were coded and scored weekly to determine the neurological end-point and incubation period of clinical disease according to previously established criteria (Bruce *et al.*, 1991).

■ **Bone-marrow reconstitution and scrapie infection.** Bone marrow (b.m.) was prepared from femurs and tibias of adult CB20 mice. Cells were suspended in Iscove's Dulbecco's medium (Gibco BRL) and a 0·1 ml volume was injected intravenously (i.v.) into the tail vein. When the intravenous injection was unsuccessful 0·1 ml was injected i.p., or the injection was termed perivenous and the volume less than 0·1 ml. Some of the cell suspension was used to count the number of viable cells; each mouse received $4{\cdot}55 \times 10^6$ viable b.m. cells. SCID mice receiving b.m. were maintained under standard specific pathogen free conditions. Five weeks post-reconstitution mice were infected i.c. with a 10^{-1} dilution of the ME7 scrapie agent. At various time-points during the incubation period animals were sacrificed and spleens harvested aseptically. Half spleens were taken for bioassay and the remainder used for immunocytochemistry.

■ **Histology/immunocytochemistry.** Spleens from ME7 scrapie-infected or uninfected SCIDs were immunolabelled with anti-PrP antibody (1B3) (McBride *et al.*, 1992), an anti-FDC monoclonal antibody (MAb) designated FDC-M1 (Koscoe *et al.*, 1992) and formal-fixed paraffin sections stained with haematoxylin and eosin (H & E). Spleens from reconstituted mice were immunolabelled with 1B3, FDC-M1, an anti-B cell MAb (B220; Caltag Laboratories) and an anti-T cell MAb (Thy-1) in order to assess the degree of reconstitution. Cryostat sections of spleen, briefly fixed in acetone, were used with the FDC-M1 and B and T cell MAbs, whereas periodate–lysine–paraformaldehyde (PLP)-fixed, paraffin-embedded sections were used with the anti-PrP antibody. Immunolabelling was carried out using the avidin–biotin complex (ABC) technique or the standard peroxidase–anti-peroxidase technique. Immunocomplex uptake following intravenous injection of HRP–anti-HRP (PAP) was performed for the recognition of functional FDCs (McBride *et al.*, 1992; Kapasi *et al.*, 1993).

■ **Infectivity assays.** SCID mice infected with the 10^{-1} dilution of scrapie by the i.c., i.p. and both s.c. routes of infection were sacrificed at 50 days post-infection (p.i.) and spleens removed for bioassay. Spleens from individual animals were prepared as 10% (w/v) homogenates in physiological saline and a 0·02 ml volume injected intracerebrally into groups of 12 recipient C3H mice. SCID mice receiving b.m. were sacrificed at 5, 10, 15 and 20 weeks post-i.c. infection. Spleens from these mice were halved for bioassay and histology. Half spleens were prepared as a 10^{-1} dilution in physiological saline and injected intracerebrally into groups of 12 C3H assay mice.

Results

ME7 infection of SCID mice

SCID and CB20 mice became affected with scrapie after ME7 i.c. infection with 10^{-2} and 10^{-3} dilutions of ME7 scrapie (Table 1), although i.p. infection at these dilutions only infected the immunocompetent CB20 mice. SCID mice infected with

Table 1. Infection of SCID and CB20 mice following i.c., i.p. or s.c. (in the neck or ventral abdomen) injection with different doses of ME7 scrapie agent

Route of infection	ME7 dilution	Scrapie incidence	Incubation period [or survival] (days)*
SCID			
i.c.	10^{-1}	11/11	155 ± 3
	10^{-2}	2/2	176 ± 8
	10^{-3}	2/2	169 ± 2
i.p.	10^{-1}	9/9	256 ± 4
	10^{-2}	0/8	[475 ± 92]
	10^{-3}	0/7	[422 ± 146]
s.c. (neck)	10^{-1}	11/11	229 ± 12
s.c. (abdomen)	10^{-1}	7/7	247 ± 13
CB20			
i.c.	10^{-2}	2/2	176 ± 8
	10^{-3}	2/2	221 ± 46
i.p.	10^{-2}	8/8	310 ± 6
	10^{-3}	8/8	333 ± 4

* Incubation periods are mean values ± SE; survival times are mean values ± SD.

Table 2. ME7 agent in spleens of SCID mice infected with a 10% dilution of scrapie brain by one of four routes of injection and harvested

The bioassay was done by i.c. injection by 10% spleen homogenate into C3H mice.

Route of infection of spleen donor	Spleen number	Scrapie incidence in assay mice	Individual incubation period in assay mice or mean ± SE (days)	Titre* (log i.c. ID_{50}/g)
i.c.	1	1/12†	260	< 2
	2	1/12‡	289	< 2
i.p.	1	11/11	236 ± 14	3·0
	2	11/12	263 ± 12	2·4
s.c. (neck)	1	0/12	–	< 2
	2	0/12	–	< 2
s.c. (abdomen)	1	0/11	–	< 2
	2	9/11	312 ± 21	< 2

* Infectivity titres calculated using a standard dose response curve for ME7.
† One animal was killed with terminal clinical signs of scrapie at 489 days p.i.; this is excluded from the numerator. The brain pathology of this animal was consistent with that of the 87A strain of scrapie not of ME7.
‡ Two animals were killed with terminal clinical signs of scrapie at 556 and 568 days p.i.; these are excluded from the numerator. Although the brain pathology of these two animals was similar to that with the ME7 scrapie strain, these incubation periods are 240–250 days longer than the longest known for ME7.

the 10^{-1} dilution became affected with scrapie using the i.c., i.p. and both s.c. routes of infection. The degenerative neuropathology in all the scrapie-affected mice was consistent with the pattern of lesion distribution produced by ME7 (Bruce *et al.*, 1991).

Infectivity bioassays of SCID spleen 50 days p.i.

There was inconsistency in the presence of scrapie agent in SCID spleens (Table 2): s.c. infection over the abdomen in two mice resulted in a low level of agent being present in one of the two spleens assayed. Agent was absent in both spleens after s.c. injection at the neck. As described in Methods some difficulty was encountered with the administration of the s.c. abdomen injections and it is probable that the injection over the abdomen in one mouse was inadvertently intraperitoneal. On the other hand, i.p. infection produced consistent but low levels of agent in both spleens with wide variation in the length of incubation periods between individual animals. After i.c. injection only traces of infectivity were found in SCID mouse spleens with a very low incidence of scrapie in assay mice. However in spleen 1, one bioassay mouse which developed clinical scrapie at 489 days p.i. was identified pathologically as a case of scrapie strain 87A, not ME7, and in spleen 2 two of the three bioassay mice which developed scrapie had incubation periods (556 and 568 days p.i.) which were much longer than those previously observed following many i.c. titrations of ME7 scrapie in C3H and other *Sinc*^{s7} mice carried out at this Unit (Fraser & Dickinson, 1985). The three cases in these bioassay mice are excluded in the numerators (Table 2).

Bioassay of bone marrow reconstituted SCID spleens

Infectivity in the spleens of reconstituted SCIDs was assessed 5, 10, 15 and 20 weeks post-i.c. infection (Table 3). High levels were present in spleens of mice receiving b.m. compared with unreconstituted SCIDs infected by the i.c. route (see Table 2). Spleens from SCID mice receiving b.m. intravenously ($n = 5$) contained high levels of infectivity with one exception, where the level of infectivity present at 140 days p.i. was lower than in the other spleens bioassayed. Spleens from SCID mice receiving b.m. intraperitoneally ($n = 5$) also contained high levels of infectivity and of the three spleens from mice which received perivenous injections, two contained low levels of infectivity, whereas one spleen contained slightly higher levels.

Spleen morphology

H & E stained sections of infected SCID spleens revealed an extreme deficiency of lymphoid structure, indistinguishable from that seen in uninfected SCID spleens. Similarly, immunostaining with MAbs to T (Thy-1) and B cells (B220) confirmed the lack of lymphoid structure. Immunolabelling with the FDC-M1 MAb in the infected and uninfected SCID spleens identified

Table 3. Titres of ME7 at 35, 70, 105 and 140 days p.i. in spleens of SCID mice infected i.c. with ME7 35 days following reconstitution with CB20 bone marrow

The bioassay was done by i.c. injection of C3H mice.

Spleen bioassay (days p.i.)	Incidence	Incubation period, (days) mean ± SE	Spleen titre (log i.c. ID_{50}/g)	Route of bone marrow injection
35	11/11	170 ± 2	5·8	i.v.
35	12/12	179 ± 2	5·2	i.p.
70	12/12	166 ± 2	6·2	i.v.
70	12/12	179 ± 3	5·2	i.v.
70	11/11	213 ± 7	3·5	Perivenous
105	12/12	178 ± 2	5·1	Perivenous
105	11/11	198 ± 4	3·9	Perivenous
105	12/12	179 ± 2	5·2	i.p.
105	12/12	182 ± 1	4·95	i.p.
140	11/11	176 ± 2	5·4	i.p.
140	12/12	173 ± 2	5·5	i.p.
140	12/12	247 ± 6	2·7	i.v.
140	12/12	181 ± 1	5·0	i.v.

some very immature and undifferentiated cells in some spleens, although there was variation in the presence and intensity of the staining of these cells between animals and even within single spleens. Immunolabelling with the anti-PrP antibody in both infected and uninfected SCIDs showed weak labelling of possible immature FDCs in some spleens, similar to that obtained with the FDC-M1 MAb. In addition, it was not possible in SCIDs to identify or define the presence of the FDCs using PAP immunocomplex uptake. In contrast, staining CB20 mouse spleen with the FDC MAb and the anti-PrP antibody revealed a normal structure. Uptake of the PAP immunocomplex and immunolabelling with the T and B antibodies also showed normal lymphoid structure.

Spleens taken from CB20 b.m. reconstituted SCIDs at 5, 10, 15 and 20 weeks after i.c. ME7 infection were assessed histologically to determine the success of bone marrow reconstitution in the spleen. SCID mice receiving b.m. intravenously had spleens which were morphologically almost indistinguishable from those of normal mice, with the exception of one spleen which had a less well developed spleen structure. Imnmunolabelling of these spleens with the MAbs to T and B cells showed the extent of the reconstitution, with well developed and strongly immunostained B-lymphoid follicles; in addition there was also strong immunostaining in the T cell areas, although these were slightly reduced in size when compared with normal animals. In addition there was strong immunolabelling with the antibody to PrP and with the FDC MAb, thus identifying the restoration of normal FDC structure. I.p. injection of b.m. also produced an effective restoration of lymphoid structure in spleens of SCIDs examined. Inadvertent perivenous b.m. injection achieved variable levels of nevertheless good reconstitution between animals. This can be shown in the titres obtained in the three spleens bioassayed from SCIDs which had received b.m. in this way (see Table 3).

Discussion

There is convincing evidence that the neuron is the primary target cell in the CNS for scrapie agent replication (Fraser & Dickinson, 1985). However, the identity of cells which permit replication of the infection in lymphoreticular and other peripheral organs is unknown (Fraser *et al.*, 1992). Whole-body exposure of mice to sublethal, lethal and supralethal doses of ionizing radiation prior to, at the time of, or during the infection has no effect on the infectibility, incubation period or pathology of the disease which suggest that dividing cells are not involved in the pathogenesis or replication of the causal agent (Fraser & Farquhar, 1987; Fraser *et al.*, 1989). This led to the suggestion that FDCs could be candidates in the peripheral replication of the agent (Fraser *et al.*, 1989) and this has been sustained by the immunolocalization of the normal host protein, PrP, which has a pivotal role in scrapie cellular pathogenesis (McBride *et al.*, 1992). FDCs are radiation-resistant (Jaroslov & Nossal, 1966) and not dependent on a high level of replenishment. Their function includes subserving B cell immunity and hence immunological memory (Koscoe *et al.*, 1992). The failure of athymia to alter scrapie pathogenesis or susceptibility is consistent with a lack of any involvement of T lymphocytes in peripheral replication, whereas the consistent and irreversible prolongation of incubation period in asplenic mice or in mice deprived of their spleens before or following scrapie infection (Fraser *et al.*, 1992; Fraser & Dickinson, 1970,

1978; Dickinson & Fraser, 1972) suggests that the candidate cells are either non-lymphoid cells but with an immune-effector function, or have no role in immunity.

In this study we have shown that there is a high dose-dependent resistance to scrapie in SCID mice following peripheral infection with the ME7 scrapie strain. SCID mice were identical to CB20 (immunocompetent) mice following i.c. infection, whereas i.p. infection of SCIDs with 10^{-2} and 10^{-3} dilutions of ME7 failed to produce disease during a 600 day observation period. However, infection with a 10^{-1} dilution resulted in 100% incidence of disease with all routes of transmission. A likely explanation is that peripheral nerve infection occurs at high dose, with consequential direct entry to the CNS without involvement of the lymphoreticular system (Kimberlin & Walker, 1988). Infectivity assays of spleens taken 50 days p.i. showed consistent but low levels of infectivity in SCIDs infected i.p., and in one of the spleens from the s.c. abdomen injected animals in which case the possibility of inadvertent i.p. injection was recorded. In contrast, no infectivity was detected in the two spleens from the s.c. neck infected animals. Trace levels of scrapie agent were detected in the two SCID spleens after i.c. infection. However, three of the five incubation periods in the C3H mice used in the bioassay were very much longer (170–250 days longer) than the longest known incubation period for the ME7 scrapie strain (Fraser & Dickinson, 1985, and unpublished). This unexpected result raises the possibility that SCIDs may have the capacity to select or amplify minor strains which hitherto have remained undetected in the extensively cloned ME7 source used (Dickinson & Outram, 1983). From spleen 1, an incubation period of 489 days occurred in a C3H bioassay mouse in which the neuropathology was indistinguishable from that associated with the 87A scrapie strain (Bruce & Dickinson, 1987). From spleen 3, incubation periods of 556 and 568 days are suggestive of the presence of an unknown strain, nevertheless with neuropathology similar to ME7. The validity of this suggestion is being tested using established strain typing procedures (Bruce *et al.*, 1991).

Bioassay of spleens indicated that while spleens taken from SCIDs injected by i.p. and s.c. (abdomen) routes contained low levels of infectivity, the i.c. and most s.c. routes produced only trace levels or no splenic infectivity. It is clear that replication is not occurring in these SCID spleens although it seems that residual inoculum remaining in the peritoneal cavity becomes passively associated with and is retained upon the spleen. This is being investigated at present by infecting, in the same way, PrP null mice ($129^{PrP-/-}$), which are resistant to scrapie infection (Bueler *et al.*, 1993; Manson *et al.*, 1994). Spleens from these animals have been assayed to determine whether adherence of scrapie has occurred following i.p. infection.

These results are inconsistent with those in a similar study in which neither disease nor even traces of splenic infection occurred following i.p. ME7 infection of SCIDs, even at high dose (O'Rourke *et al.*, 1994). In the latter, bioassay was undertaken using centrifuged spleen supernates which leads to loss of titre (unpublished observation).

In contrast, bioassay of spleens taken from the i.c. infected b.m. reconstituted SCIDs at 5 to 20 weeks, in which reconstitution was i.v. or i.p., produced 100% incidence of disease in assay mice with incubation periods consistent with replication to high titre. The morphology of these spleens, in terms of FDC structure and B and T cell presence, was almost indistinguishable from those of the normal mice examined. In the bioassay of three spleens in which reconstitution was perivenous there was variability in the amounts of infectivity present which correlated with the success of b.m. reconstitution in these spleens. These results show that the amount of infectivity present in spleen is related to the success of the reconstitution, in terms of normal lymphoid structure and in the structure and presence of FDCs.

These results indicate that replication of ME7 scrapie occurs only in SCID spleens in which b.m. reconstitution has been successful. Although this does not eliminate other interpretations it supports other data suggesting that FDCs are permissive for scrapie replication. The SCID mouse model is being used in further experiments the aim of which is to confirm or exclude the FDC as the specific cell type responsible for replication of scrapie in the spleen.

The authors would like to acknowledge the gift of the monoclonal antibody to follicular dendritic cells supplied by Dr Marie Koscoe of the Basel Institute of Immunology and the gift of the monoclonal antibody Thy-1 by Dr George Kraal of the Free University of Amsterdam. We would also like to acknowledge Dr Jan Fraser for her assistance and also Dawn Drummond for technical assistance.

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Received 26 March 1996; Accepted 19 April 1996