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Abstract

An intact immune system, and particularly the presence of mature B lymphocytes, is crucial for mouse scrapie pathogenesis in the brain after peripheral exposure. Prions are accumulated in the lymphoreticular system (LRS), but the identity of the cells containing infectivity and their role in neuroinvasion have not been determined. We show here that although prion infectivity in the spleen is associated with B and T lymphocytes and to a lesser degree with the stroma, no infectivity could be detected in lymphocytes from blood. In wild-type mice, which had been irradiated and reconstituted with PrP-deficient lymphohaematopoietic stem cells and inoculated with scrapie prions, infectivity in the spleen was present in the stroma but not in lymphocytes. Therefore, splenic B and T lymphocytes can either synthesize prions or acquire them from another source, but only when they express PrP.
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An intact immune system, and particularly the presence of mature B lymphocytes, is crucial for mouse scrapie pathogenesis in the brain after peripheral exposure. Prions are accumulated in the lymphoreticular system (LRS), but the identity of the cells containing infectivity and their role in neuroinvasion have not been determined. We show here that although prion infectivity in the spleen is associated with B and T lymphocytes and to a lesser degree with the stroma, no infectivity could be detected in lymphocytes from blood. In wild-type mice, which had been irradiated and reconstituted with PrP-deficient lymphohaematopoietic stem cells and inoculated with scrapie prions, infectivity in the spleen was present in the stroma but not in lymphocytes. Therefore, splenic B and T lymphocytes can either synthesize prions or acquire them from another source, but only when they express PrP.

Keywords: B lymphocytes/follicular dendritic cells/prions/PrP knockout mice/T lymphocytes

Introduction

Intracerebral (i.c.) inoculation of mice with scrapie agent results in a rise of infectivity in the spleen, weeks or months before the brain is affected. However, in the case of non-experimental forms of spongiform encephalopathies, such as sheep scrapie, BSE or iatrogenic forms of Creutzfeldt–Jakob disease (CJD), the agent usually enters from the periphery; intraperitoneal (i.p.) inoculation of mice with scrapie prions is therefore a more realistic model. This procedure results in rapid accumulation of infectious agent in the spleen and other tissues of the lymphoreticular system (LRS) (Ekuldd et al., 1967; Pattison and Jones, 1968; Fraser and Dickinson, 1970; Mould et al., 1970), following which prions are probably transported along tracts of the peripheral nervous system to enter the brain, presumably via the spinal cord (Kimberlin and Walker, 1980, 1988, 1989; Beekes et al., 1996) or the N.vagus (Beekes et al., 1998). Transport from the periphery to the central nervous system (CNS) depends on elements of the LRS, as evidenced by the fact that development of CNS disease after i.p. inoculation with scrapie is impaired or abolished in mice with various forms of immunodeficiency, such as SCID, RAG–/– or μMT mice, while i.c. inoculation continues to be fully effective (O’Rourke et al., 1994; Fraser et al., 1996; Lasmezas et al., 1996; Taylor et al., 1996; Brown et al., 1997; Klein et al., 1997).

Results

Determination of scrapie infectivity in peripheral blood leukocytes and fractionated splenocytes

In a first experiment, we analysed spleens of wild-type (129/Sv-C57BL/6) mice 34 days after i.p. inoculation with RML prions. Spleens were fractionated into pulp and stroma. B and T cells were purified from the pulp fraction by magnetic-activated cell sorting (MACS) followed by complement lysis of B cells in the T-cell fraction and vice versa. Finally, viable cells were isolated by density gradient centrifugation. This three-step procedure led consistently to highly purified T- and B-lymphocyte preparations devoid of detectable cross-contamination, as shown by fluorescence-activated cell sorting (FACS) analysis (Figure 1), in ~5–10% yield. In addition, a non-B, non-T cell population was obtained by depleting splenocytes of B and T lymphocytes by MACS; this fraction contained ~2% T but no detectable B lymphocytes. The cell preparations and the stroma fraction were analysed for infectivity by endpoint titration (Brandner et al., 1996; Fischer et al., 1996) (Table I). Total splenocytes had ~3.5 log LD50 units per 106 cells, and both B and T cells showed infectivity titres within the same order of magnitude, 3.4 and 3.5 log LD50 units per 106 cells, respectively. Strikingly, the non-B, non-T cell population contained only ~1 log LD50 unit per 106 cells (which could be attributed to the ~2% contamination by T lymphocytes), arguing that prion infectivity in the splenocyte fraction was not due to unspecific contamination with infectivity released from the stromal fraction (Clarke and Kimberlin, 1984). The pulp of a spleen contains ~2×108 cells, ~80% of which are lymphocytes (Binder et al., 1997). Inasmuch as the purified B and T cells were representative of their class as regards infectivity, ~300×3.5 log LD50 units = 6 log LD50 units of infectivity were associated with the pulp and ~4.3 log LD50 units with the stroma of one spleen (Figure 2). Essentially all infectivity detected in total spleen extracts was accounted for by the fractions. There was ~50 times more infectivity associated with lymphocytes than with stroma. Clarke and Kimberlin (1984) have reported about equal distribution of infectivity between pulp and stroma; however, their data were obtained with mouse and scrapie strains different from ours.

These findings suggested that lymphocytes might be responsible for spreading prions through the organism. We therefore determined the infectivity of peripheral blood leukocytes (PBLs) from the same animals whose spleens had been analysed. We were surprised to find that despite
Association of prions with splenic lymphocytes

Fig. 1. Flow cytometric analysis of splenocytes and purified splenocyte fractions. Splenocytes from wild-type mice 34 days after i.p. inoculation with RML scrapie agent were fractionated as described in the Materials and methods, and subjected to FACS analysis. More than 99% of the cells in the purified B cell fraction were positive for the B-cell marker B220 and negative for the T-cell marker CD3. Similarly, >99% of purified cells in the T-cell fraction were positive for CD3 and negative for B220. The same results were obtained whether or not the cells were gated for lymphocytes by forward and side scattering. Ordinate, cell counts; abscissa, logarithm of fluorescence intensity.

Table I. Infectivity in spleen cell fractions and PBLs of scrapie-infected mice

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Cell No. or dilution</th>
<th>Tg94/IRF</th>
<th>Prnp&lt;sup&gt;+/−&lt;/sup&gt;</th>
<th>Prnp&lt;sup&gt;+/−&lt;/sup&gt;[FLC-Prnp&lt;sup&gt;0/0&lt;/sup&gt;]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Inc. time</td>
<td>n/No.</td>
<td>Inc. time</td>
</tr>
<tr>
<td>Splenocytes</td>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>n.d.</td>
<td>n.d.</td>
<td>&gt;200</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>88, 90</td>
<td>(2/2)</td>
<td>81, 86</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>106.5 ± 18</td>
<td>(4/4)</td>
<td>89 ± 5</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>108, 125</td>
<td>(2/4)</td>
<td>106 ± 7</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>&gt;200</td>
<td>(0/4)</td>
<td>&gt;200</td>
</tr>
<tr>
<td>B cells</td>
<td>2 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>n.d.</td>
<td>n.d.</td>
<td>&gt;200</td>
</tr>
<tr>
<td></td>
<td>2 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>106 ± 10</td>
<td>(4/4)</td>
<td>88 ± 2</td>
</tr>
<tr>
<td></td>
<td>2 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>98, 114</td>
<td>(2/4)</td>
<td>102 ± 14</td>
</tr>
<tr>
<td></td>
<td>2 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>&gt;200</td>
<td>(0/4)</td>
<td>91</td>
</tr>
<tr>
<td>T cells</td>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>n.d.</td>
<td>n.d.</td>
<td>&gt;200</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>109, 110</td>
<td>(2/4)</td>
<td>94 ± 8</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>&gt;200</td>
<td>(0/4)</td>
<td>95, 121</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>&gt;200</td>
<td>(0/4)</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Non-B/T cells</td>
<td>5 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>108</td>
<td>(1/4)</td>
<td>112 ± 14</td>
</tr>
<tr>
<td></td>
<td>5 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>n.d.</td>
<td>n.d.</td>
<td>&gt;200</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>&gt;200</td>
<td>(0/4)</td>
<td>&gt;200</td>
</tr>
<tr>
<td>PBL</td>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>&gt;200</td>
<td>(0/4)</td>
<td>&gt;200</td>
</tr>
<tr>
<td></td>
<td>undiluted</td>
<td>69 ± 2</td>
<td>(4/4)</td>
<td>72 ± 3</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;−2&lt;/sup&gt;</td>
<td>80, 94</td>
<td>(2/4)</td>
<td>108 ± 20</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;−4&lt;/sup&gt;</td>
<td>&gt;200</td>
<td>(0/4)</td>
<td>&gt;200</td>
</tr>
</tbody>
</table>

*Wild-type mice (Prnp<sup>+/−</sup>), transgenic Prnp<sup>0/0</sup> mice overexpressing PrP in spleen (Tg94IRF) and wild-type mice irradiated and reconstituted with FLCs from Prnp<sup>0/0</sup> mice (Prnp<sup>+/−</sup>[FLC-Prnp<sup>0/0</sup>]) were inoculated i.p. with RML prions. Spleens were recovered after 34 days and processed as described in the Materials and methods section. The number of cells indicated or a 1% stroma homogenate at the dilution indicated was inoculated intracerebrally into indicator mice and the time to disease (inc. time) was recorded. n/No., number of animals succumbing to scrapie/number inoculated. n.d., not done.
Association of infectivity with splenic lymphocytes is dependent on PrP expression

We have shown previously that prion replication does not occur in the spleen of Prnp<sup>0/0</sup> mice (Bueler et al., 1993). Moreover, reconstitution of the LRS of Prnp<sup>0/0</sup> mice with PrP-expressing fetal liver cells (FLCs) restored prion accumulation in the spleen (Bla¨ ttler et al., 1997). To assess whether the association of infectivity with lymphocytes was specific or adventitious, we lethally irradiated Prnp<sup>+/+</sup> mice and reconstituted them with FLCs derived from Prnp<sup>0/0</sup> mice. PCR analysis of splenocytes, PBLs, stroma and tail tissue confirmed that these mice had undergone successful reconstitution, and FACS analysis of lymphocytes demonstrated the Prnp<sup>0/0</sup> origin of these cells (data not shown). Spleens from these mice, 34 days after i.p. inoculation with RML prions, were fractionated and analysed: no infectivity was found in either total splenocytes (<1 LD<sub>50</sub> unit per 10<sup>6</sup> cells), or in purified B or T lymphocytes (<1 LD<sub>50</sub> unit per 10<sup>5</sup> cells). However, prion titres in the stromal fraction were not significantly different from those in wild-type mice (Table I; Figure 2). Two-colour immunofluorescence analysis of spleen sections stained with follicular dendritic cell marker FDC-M1 and a polyclonal antibody to PrP revealed PrP accumulation in FDCs (Klein et al., 1998). From these results, we conclude that scrapie infectivity in spleens of irradiated mice reconstituted with PrP<sup>0/0</sup> lymphohaeopoietic stem cells is probably associated with FDCs and that splenic lymphocytes devoid of PrP fail to produce or to take up scrapie infectivity.

Discussion

It is remarkable that despite the >1000-fold overexpression of PrP in the spleen of Tg94/IRF mice, the prion titre in this...
organ was not higher than that in wild-type mice. Either the level of PrP is not the limiting factor for the formation of prions or else overexpression is not occurring in the cells in which prions are formed. Similarly, at the terminal stage of scrapie, the prion titrue in brains of tga20 mice, which overexpress PrP 5- to 8-fold, is about the same as in wild-type, although the incubation times for the transgenic and wild-type mice are 60 and 160 days, respectively (Fischer et al., 1996).

The experiments described here show that in the spleen of i.p. scrapie-infected wild-type mice, prions are associated with B and T lymphocytes as well as with the stroma, but not with the pulp-derived non-B, non-T cell fraction which consists mainly of monocytes and granulocytes (Binder et al., 1997). Moreover, there was <1 LD50 unit of infectious agent in ~106 lymphocytes recovered from peripheral blood, i.e. the specific infectivity of lymphocytes in PBLs was at least 2000-fold lower than in the spleen. Because a mouse has ~2×107 circulating lymphocytes, there would be <20 LD50 units of cell-associated infectivity, if any, circulating at one time.

Were the prions associated with splenic lymphocytes generated in these cells or were they acquired from other sources? Unspecific contamination is unlikely because the specific infectivity of non-B, non-T cells derived from the pulp was 2000–3000 times less than that of B or T lymphocytes and because B and T lymphocytes devoid of PrP failed to acquire infectivity in a spleen whose stroma did contain infectious agent. We have generated mice expressing PrP exclusively on T cells (Raebet et al., 1996) or on B cells (F.Montrasi, A.J.Raebet and C.Weissmann, unpublished results) and i.p. inoculation of such mice with scrapie agent failed to result in disease or accumulation of infectivity in the spleen. If lymphocytes are at all able to replicate scrapie agent, then perhaps they can do so only in the context of other types of PrP-bearing cells.

Infectivity in the stromal component of peripheral lymphoid organs is thought to reside in radiation-resistant post-mitotic cells (Fraser and Farquhar, 1987; Fraser et al., 1989). A prime candidate is the follicular dendritic cell, because the pathological isoform of the prion protein, PrPSc, co-localizes with FDCs in mice inoculated with CJD or scrapie agent (Kitamoto et al., 1991; McBride et al., 1992; Muramoto et al., 1992). Because lymphocytes interdigitate with FDCs (Heinen et al., 1995), they might have acquired prions or prion-containing, torn-off membrane fragments from the latter during the isolation procedure. However, when wild-type mice were irradiated and reconstituted with PrP-less FLCS, no infectivity was found on splenic lymphocytes, which means that if this ‘transfer’ hypothesis is correct, the postulated adhesion of scrapie agent is dependent on the presence of PrP on lymphocytes; PrP would then function as the receptor for the infectious agent. A likely source of prions would be the FDCs.

Why is infectivity found on splenic but not on circulating lymphocytes? Perhaps only distinct subsets of splenic lymphocytes carry infectivity and these do not enter the blood stream, or perhaps prion-carrying splenic lymphocytes are eliminated in the spleen. Alternatively, prions may attach to lymphocytes only during the preparation procedure, albeit through a PrP-dependent interaction.

As regards the role of lymphocytes in the spread of infectivity from the periphery to the CNS, it would seem that circulating, prion-bearing B cells are not required, not only because they were not detected outside the LRS, but because reconstitution of irradiated wild-type mice (Blattler et al., 1997) or immunodeficient mice (Klein et al., 1998) with LRS devoid of PrP restored invasion of the CNS by prions following i.p. inoculation.

Materials and methods

**Scrapie infection**

RML is a mouse-adapted scrapie isolate (Chandler, 1961). It was passaged in Swiss CD-1 mice obtained from Charles River Laboratories. Inocula were 10% (w/v) homogenates of RML-infected CD-1 mouse brains in 0.32 M sucrose. Mice were infected i.p. with 100 μl of a 10-fold dilution of the inoculum in phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA).

**Bone marrow reconstitution**

Eight-week-old Prnp+/− mice (129/SvxC57BL/6) were lethally irradiated and reconstituted with FLCS from E14.5–15.5 Prnp0/0 (129/SvxC57BL/6) embryos as described previously (Blattler et al., 1997). The extent of reconstitution was assessed by FACS and PCR 6–8 weeks after grafting. Inoculation with mouse scrapie prions was carried out 12 weeks after reconstitution.

**Preparation of splenocytes and stroma**

Spleens were collected from mice 34 days after i.p. inoculation with the RML strain of prions. Splenocytes were obtained by forcing spleens through a fine mesh platinum wire net. Cell suspensions were prepared in PBS with 1% BSA, 5 mM EDTA and 0.01% sodium azide (MACS buffer). The stromal fraction was recovered from the net and a 1% (w/v) homogenize in PBS-5% BSA was prepared by forcing the suspension through an 18 gauge needle. The homogenate was digested with 1 mg/ml collagenase D (Boehringer Mannheim) for 60 min at 37°C and sonicated (Branson Sonifier, maximal output) four times for 1 min.

**B and T cell purification**

Splenocytes were incubated with anti-mouse B220 or Thy1.2 antibodies conjugated with super-paramagnetic microbeads (Milteny Biotec GmbH, Germany) for 15 min at 4°C and applied to a pre-filled and washed A2 column fixed onto the VARIO MACS (Milteny Biotec GmbH). Unlabelled cells were eluted with MACS buffer using a 23 gauge syringe attached to the column outlet as flow resistor. The column was removed from the magnet and cells were backflushed using a syringe attached to the column outlet. The column was fixed to the magnet and the cell suspension was allowed to enter the column. Unlabelled cells were again rinsed out with MACS buffer. Finally, the column was removed from the magnet and labelled cells were eluted by rinsing the column with MACS buffer.

**B and T cell depletion**

Splenocytes were incubated with anti-mouse B220 and anti-mouse Thy1.2 antibodies conjugated with super-paramagnetic microbeads for 15 min at 4°C and applied to a CS column fixed onto the VARIO MACS (Milteny Biotec GmbH). Unlabelled cells were eluted with MACS buffer as described above. The flow-through fraction was once again loaded onto a CS column and unlabelled cells eluted in MACS buffer.

**Complement lysis**

MACS-purified B- and T-cell fractions were purified further by complement lysis of B cells in the T-cell fraction and vice versa. Cells were pelleted and resuspended in RPMI-1640 with 25 mM HEPES (pH 7.4) and 0.3% BSA [cytotoxicity medium (CM)] to give 1–3×107 cells/ml. For B cell depletion, cells were incubated with a 1:200 dilution of rat anti-mouse LR1 antibody (clone LR6.2B6D6.C9, Serotec). For T cell depletion, cells were incubated with a 1:400 dilution of mouse anti-Thy1.2 antibody (clone F7D5, Serotec) at 4°C for 60 min. The cells were resuspended to the original density in CM containing 20% Low-tox-M rabbit complement (Cedarlane, Ontario, Canada) and incubated for 60 min at 37°C. Viable lymphocytes were separated from dead cells and debris by centrifugation over Lympholyte-M as recommended by the manufacturer (Cedarlane, Ontario, Canada).
Isolation of PBLs from whole blood

PBLs were isolated from whole blood by density gradient centrifugation using Lympholyte-M as recommended by the manufacturer (Cedarlane, Ontario, Canada).

FACS analysis

Single-cell suspensions were prepared in PBS, 2% fetal calf serum, 20 mM EDTA, 0.01% sodium azide (FACS buffer). For flow cytometry, cells were stained with saturating concentrations of fluorescein-conjugated antibodies (1 µg/10^6 cells) for 30 min at 4°C and washed in FACS buffer. Data acquisition and analysis were performed with an EPICS XL (Couler) flow cytometer. Dead cells were gated out by forward and side scatter properties. Monoclonal antibodies used were fluorescein isothiocyanate (FITC)-conjugated RA3-6B2 (B220) (Gibco-BRL) and FITC-conjugated KT3 (CD5) (Serotec).

Determination of infectivity

Cell fractions were collected by centrifugation for 10 min at 1000 rpm and diluted serially in PBS–5% BSA. Tg20 mice (Fischer et al., 1996) were inoculated i.c. with 30 µl of inoculum into the right parietal lobe, using a 26 gauge hypodermic needle. Prion titres were estimated from the incubation times (Brandner et al., 1996) and/or dilution endpoints (Reed and Muench, 1938).

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References


