Immunological differences between susceptible and resistant sheep during the preclinical phase of scrapie infection


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In order to investigate the relationship between the immune response to scrapie infection and genetic susceptibility to the disease in sheep, immune cell subsets and prion protein (PrP) expression were determined in susceptible and resistant Suffolk sheep in the preclinical phase of infection. At 6 months of age, 12 ARQ/ARQ (susceptible) and nine ARR/ARR (resistant) scrapie-free Suffolk lambs were challenged subcutaneously with scrapie inoculum. Prefemoral lymphadenectomies were carried out at 14 and 180 days post-inoculation (p.i.) and serial bleeds were collected at monthly intervals for up to 1 year p.i. An indirect double-labelling procedure was carried out on peripheral blood mononuclear cells (PBMCs) and lymph node cell preparations and analysed using flow cytometry. Prior to scrapie challenge, significantly more PrP⁺ cells were detected in PBMCs from the susceptible sheep. Furthermore, following challenge, significantly more CD8⁺ and γδ⁺ T cells were detected in the PBMCs of the resistant sheep. However, at both 14 and 180 days p.i., CD21⁺ cell expression was significantly higher in the lymph node preparations of the susceptible sheep. In contrast, more CD4⁺ cells were detected in the lymph nodes of the resistant sheep at both time points. It was concluded that significant differences in immune cell subsets and PrP expression occur between ARQ/ARQ and ARR/ARR Suffolk sheep in the preclinical phase of infection.

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) are a group of neurodegenerative diseases affecting mammalian species including sheep, deer, cattle and humans (Hur et al., 2002). Scrapie is a TSE that affects sheep and goats and has been recorded in Great Britain since the mid-18th century (Parry, 1983). Long incubation periods preceding disease are a hallmark of TSEs, yet tissues can be infective before the advent of clinical signs (Carp et al., 1989; Hadlow et al., 1982).

The causative agent and route of infection remain elusive; however, the abnormal isoform of the cellular prion protein (PrPSc), designated PrPSc, is considered to be a disease-associated marker (Prusiner, 1982). In most natural and experimental cases of scrapie, PrPSc is present initially in lymphoreticular tissue prior to accumulating in the central nervous system (Kimberlin & Walker, 1979; Hadlow et al., 1982; Lasmezas et al., 1996; van Keulen et al., 2002).

Comparative studies of naturally infected sheep and sheep infected subcutaneously with the scrapie isolate SSBP/1 have shown a strong association between clinical signs, deposition of PrPSc in the tissues and vacuolation in the brain (Houston et al., 2002). However, PrP detection and changes in immune system cells during the preclinical phase of scrapie infection are not well documented in sheep and require further investigation.

The breed and PrP genotype are important in assessing the susceptibility of sheep to scrapie (Hunter et al., 1997). With Suffolk sheep, the genotype ARQ/ARQ is most susceptible and ARR/ARR is most resistant. It is unclear, however, why these polymorphisms are so influential in the susceptibility status of the animals (Baylis & Goldmann, 2004). There is no known link between susceptibility to scrapie in sheep and efficiency of the immune response of the host and this therefore remains another intriguing question to be answered.
Experimental design. Twelve ARQ/ARQ (susceptible) and nine ARR/ARR (resistant) female or castrated male Suffolk lambs were obtained from a DEFRA scrapie-free flock (Houston et al., 2002). At 6 months of age, each lamb received 1 ml of 10% clarified Suffolk ARQ/ARQ scrapie brain pool homogenate (see below) injected subcutaneously into the drainage area of the right prefemoral lymph node. The lambs were divided into two groups determined by the time of prefemoral lymphadenectomy, with six susceptible and four resistant lambs in group 1 and six susceptible and five resistant lambs in group 2. The ipsi- and contralateral prefemoral lymph nodes were removed surgically from group 1 animals at 14 days post-inoculation (p.i.) and from group 2 animals at 180 days p.i. Serial bleeds were carried out at monthly intervals for up to 1 year post-inoculation for use in flow cytometry.

All animal procedures complied with the Animals (Scientific Procedures) Act 1986 and were approved by the ethics committee at the Moredun Research Institute, UK.

Inoculum preparation. A pool of brains from clinical cases of Suffolk scrapie (ARQ/ARQ) from a naturally infected flock, confirmed positive by immunohistochemistry (González et al., 2002), was homogenized in four times their volume of 0.32 M sucrose solution to produce a 20% homogenate. The pooled homogenate was then centrifuged at 850 g for 20 min and the clarified homogenate was diluted further by the addition of an equal volume of 0.32 M sucrose to give a 10% solution.

Sample collection. Fresh whole blood was obtained by jugular venipuncture and collected in lithium heparin-coated tubes. The ipsi- and contralateral prefemoral lymph nodes were removed and a representative sample of each node was collected into Hanks’ balanced salts solution (HBSS) with 5% heat-inactivated fetal calf serum (FCS) and penicillin/streptomycin (100 U ml⁻¹ and 100 μg ml⁻¹, respectively).

Flow cytometry. Cell suspensions. The samples of lymph node were minced finely in HBSS solution supplemented with FCS and penicillin/streptomycin as above and disaggregated with a Stomacher 80 Biomaster (Seward) for 120 s at high power before being filtered through lens tissue paper (Whatman 10 x 150 mm). Red cells from the lymph node cell preparations and heparinized blood were lysed by mixing sterilized Tris/NH₄Cl solution (0.144 M NH₄Cl, 0.017 M Tris/HCl, pH 7.65) at 37 °C with an equal volume of blood. Cells were then counted using a cell counter (Beckman Coulter) and their concentration adjusted to 1 x 10⁶ cells ml⁻¹.

Cell labelling. An indirect double-labelling procedure was carried out on the blood and lymph node samples as described previously (Smith et al., 1994). Using the mAbs listed in Table 1, cells were first labelled with a combination of cluster domain (CD)-specific antibody and an antibody raised against either the N-terminal residues 23–90 of PrP (FH11) or ovine MHC class II (SW73.2). The CD markers were detected with an anti-mouse IgG1 antibody conjugated to Alexa Fluor 488 (Cambridge Biosciences) and mAbs FH11 and SW73.2 were detected with an anti-mouse IgG2b antibody conjugated to R-phycocerythrin and an anti-rat IgG conjugated to R-phycocerythrin, respectively (Caltag MedSystems). Unconjugated isotype-matched irrelevant control mAbs were included in all experiments. All incubation steps were carried out at 4 °C for 30 min and cells were washed (to remove excess unbound antibody) between incubations with PBS containing 5% FCS and 0.02% sodium azide. Cells were fixed in 1% paraformaldehyde in PBS and stored in the dark at 4 °C until analysed.

Cell acquisition and analysis. Data were acquired on a FACSCalibur flow cytometer and analysed using CellQuest software (both from Becton Dickinson). The live gates were set on a linear forward/side scatter axis, which allowed elimination of debris and dead cells from the analysis and gating of the peripheral blood mononuclear cells (PBMCs). At least 10 000 gated cells were acquired for each sample. The fluorescence of the selected lymphocyte population was analysed on a logarithmic scale, with results expressed as the percentage of the gated double-labelled cells.

Follow-up of infection. Tonsil biopsy samples (Jeffrey et al., 2001) were collected at 1 year intervals after infection until sheep were...
Table 1. Reactivity of mAbs

<table>
<thead>
<tr>
<th>mAb</th>
<th>Isotype</th>
<th>Target antigen</th>
<th>Target cell/protein</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FH11</td>
<td>IgG2b</td>
<td>PrP N-terminal residues 23–90</td>
<td>Prion protein</td>
<td>Foster et al. (1996)</td>
</tr>
<tr>
<td>17D</td>
<td>IgG1</td>
<td>Ovine CD4</td>
<td>T-helper cells</td>
<td>Mackay et al. (1988)</td>
</tr>
<tr>
<td>86D</td>
<td>IgG1</td>
<td>Ovine γδ TCR</td>
<td>γδ T cells</td>
<td>Mackay &amp; Hein (1989)</td>
</tr>
<tr>
<td>CC21</td>
<td>IgG1</td>
<td>Bovine CD21</td>
<td>B cells/CR2</td>
<td>Naessens et al. (1997)</td>
</tr>
<tr>
<td>ILA-51</td>
<td>IgG1</td>
<td>Ovine CD8</td>
<td>Cytotoxic T cells</td>
<td>Naessens et al. (1997)</td>
</tr>
<tr>
<td>VPM65</td>
<td>IgG1</td>
<td>Ovine CD14</td>
<td>Macrophages, monocytes</td>
<td>Gupta et al. (1996)</td>
</tr>
<tr>
<td>SW73.2</td>
<td>IgG2b</td>
<td>Ovine MHC class II, β-chain</td>
<td>B cells, activated T cells, macrophages, monocytes, dendritic cells</td>
<td>Hopkins et al. (1986)</td>
</tr>
</tbody>
</table>

confirmed as PrPSc-positive by immunohistochemistry (González et al., 2002).

**Statistical methods.** For the lymph node data, a mixed-effect model was used with genotype, node of challenge and time of lymphadenectomy included as fixed effects and sheep as a random effect. The statistical analyses were implemented within Microsoft Excel. Apart from the false discovery rate (FDR) procedure, which was taken using Genstat (version 8 for Windows; VSN International), carried out on both sets of data. All statistical analyses were undertaken using Genstat (version 8 for Windows; VSN International), apart from the false discovery rate (FDR) procedure, which was implemented within Microsoft Excel.

**RESULTS**

At 1 year p.i., nine out of 12 ARQ/ARQ sheep were PrPSc-positive by tonsil biopsy and by 2 years p.i. one further ARQ/ARQ sheep was confirmed to be positive. The two ARQ/ARQ animals with negative tonsil biopsy results were clinically normal when culled at 18 months p.i. All samples from ARR/ARR sheep were negative for PrPSc by immunohistochemistry in tonsil biopsies at 12 and 24 months p.i.

The results given below only describe the statistically significant findings; however, every cell subset dual-labelled with either PrP or MHC II was analysed.

Phenotyping of prefemoral lymph node cells at 14 and 180 days p.i.

PrP surface labelling was detected on each cell phenotype investigated (CD4+, CD8+, γδ T cell, CD21+ and CD14+) within the prefemoral lymph node population. In the analyses, for each cell phenotype, only those effects that had an associated P value of less than 0.02 were judged to be of interest and these are summarized in Table 2. This cutoff point for the P value was based on an FDR of 20%. The FDR was set at a relatively high level so that potentially interesting results were not excluded. However, this meant that some of the apparent effects could have arisen by chance.

**CD21+ cell population**

The mean percentages of the CD21+ cell population from the susceptible ARQ/ARQ sheep were significantly higher than those from the resistant animals in the ipsi- and contralateral lymph nodes at 14 and 180 days p.i. (mean difference of −9%, P < 0.001). The challenged node was found to have significantly more CD21+ cells (P = 0.002) than the unchallenged node. A similar pattern of results was observed for the dual-labelled CD21+/MHC class II+ cell percentages (Table 2; Fig. 1a–h).

**CD4+ cell population**

A significant correlation between PrP genotype, days p.i. and lymph node (challenged or not) was detected in the single-labelled CD4+ cells (P = 0.006) from the resected lymph nodes. A higher percentage of CD4+ cells was detected in resistant sheep than in susceptible sheep at both 14 and 180 days p.i. The greatest difference in the CD4+ cells was detected at 14 days p.i., with 10% more CD4+ cells detected in resistant sheep (estimated mean of 59%) when comparing the resistant and susceptible animal unchallenged lymph node cell preparations. At 180 days p.i., the percentage of CD4+ cells was found to be higher in the challenged node of resistant sheep, although in the unchallenged node similar percentages of CD4+ cells were detected in both genotypes.

**CD8+/PrP+ cells**

The mean percentage of cells dual-labelled with CD8 and PrP was found to be significantly higher in the unchallenged than in the ipsilateral lymph node cells.
(P = 0.019), at 14 and 180 days p.i., in both susceptible and resistant sheep.

Peripheral blood phenotyping
PrP was detected on every cell type tested (CD4⁺, CD8⁺, γδ T cell, CD21⁺ and CD14⁺) in the mononuclear fraction of the blood. The pattern of responses over time for PrP⁺ cells, singly or doubly labelled with any of the immune cell subset markers, appeared to be influenced by time post-inoculation and PrP genotype. Three of these cell phenotypes that appeared to be significantly influenced by genotype are illustrated in Fig. 2(a–c). According to the FDR analysis, the observed differences in the pattern of

Table 2. Estimated mean percentages of positive cells from resected lymph nodes of susceptible and resistant sheep

<table>
<thead>
<tr>
<th>Marker</th>
<th>Genotype</th>
<th>Lymph node</th>
<th>Mean SEM</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Challenged</td>
<td>Unchallenged</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>14 days p.i.</td>
<td>180 days p.i.</td>
<td>14 days p.i.</td>
</tr>
<tr>
<td>CD4</td>
<td>ARQ</td>
<td>48.8</td>
<td>50.5</td>
<td>48.7</td>
</tr>
<tr>
<td></td>
<td>ARR</td>
<td>51.6</td>
<td>55.6</td>
<td>59.4</td>
</tr>
<tr>
<td>CD4/MHC II</td>
<td>ARQ</td>
<td>9.1</td>
<td>6.9</td>
<td>9.3</td>
</tr>
<tr>
<td></td>
<td>ARR</td>
<td>12.5</td>
<td>19.1</td>
<td>17.4</td>
</tr>
<tr>
<td>CD8/MHC II</td>
<td>ARQ</td>
<td>8.6</td>
<td>5.3</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>ARR</td>
<td>6.4</td>
<td>2.6</td>
<td>8.6</td>
</tr>
<tr>
<td>CD8/prion</td>
<td>ARQ</td>
<td>4.5</td>
<td>3.0</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>ARR</td>
<td>5.0</td>
<td>3.4</td>
<td>5.5</td>
</tr>
<tr>
<td>CD21</td>
<td>ARQ</td>
<td>28.1</td>
<td>28.2</td>
<td>24.3</td>
</tr>
<tr>
<td></td>
<td>ARR</td>
<td>21.1</td>
<td>12.6</td>
<td>15.1</td>
</tr>
<tr>
<td>CD21/MHC II</td>
<td>ARQ</td>
<td>25.2</td>
<td>25.4</td>
<td>22.7</td>
</tr>
<tr>
<td></td>
<td>ARR</td>
<td>20.5</td>
<td>12.3</td>
<td>14.9</td>
</tr>
</tbody>
</table>

*Only cell-types for which there was evidence of a genotype or treatment effect (P < 0.02) are included in the table. g denotes the P value associated with the effect of genotype, c the effect of challenge and t the effect of time. g × t × c is the P value associated with the three-way interaction.
responses between the two genotypes were likely to be
genuine differences, even though the results for different
markers were not all independent of each other.

**PrP**<sup>+</sup> cells

Prior to scrapie challenge, the percentage of single-labelled
PrP<sup>+</sup> cells was significantly higher in the susceptible
(estimated mean of 60%) compared with the resistant
(mean of approximately 40%, P < 0.001; Fig. 2a) sheep.
Similar differences were also detected in CD4<sup>+</sup>, CD8<sup>+</sup> and
CD14<sup>+</sup> cells (P < 0.001) dual-labelled with PrP. After chal-
lenge, the percentage of both the susceptible and resistant
sheep PrP<sup>-</sup>-labelled PBMCs remained relatively constant,
at approximately 40%, for the 9 month period p.i.

**γδ T-cell receptor (TCR)<sup>+</sup>/PrP<sup>+</sup> cells**

The percentage of γδ T cells dual-labelled with PrP (Fig.
2b) was very similar for both the susceptible and resistant
sheep (mean of approximately 7%) prior to inoculation.
After inoculation, the percentage of γδ TCR<sup>+</sup>/PrP<sup>+</sup> cells
decreased significantly to an estimated mean of 2% in the
susceptible sheep, whereas levels remained constant in
the resistant animals up to 2 months p.i. After 2 months,
the γδ TCR<sup>+</sup>/PrP<sup>+</sup> cell percentages in the resistant sheep
declined steadily until 5 months p.i. when the estimated
mean percentage of γδ TCR<sup>+</sup>/PrP<sup>+</sup> cells for both geno-
types was approximately 2%. From 5 to 9 months p.i.,
the mean percentage of γδ TCR<sup>+</sup>/PrP<sup>+</sup> cells remained fairly
constant in the susceptible and resistant sheep.

**CD8<sup>-</sup>/PrP<sup>+</sup> cells**

A significant interaction (P < 0.001) between PrP genotype
and time was detected in the CD8<sup>-</sup>/PrP<sup>+</sup> dual-labelled
PBMCs (Fig. 2c). Before inoculation, the mean percentage
of CD8<sup>-</sup>/PrP<sup>+</sup> cells was significantly higher in the
susceptible sheep with a mean of 11% compared with a
mean of 3% in the resistant sheep. After challenge with the
scrapie-infected inoculum, the percentage of CD8<sup>-/PrP<sup>+</sup></sup>-
labelled cells detected decreased in the susceptible animals
to a mean of 7%. Conversely, the mean percentage of
CD8<sup>-/PrP<sup>+</sup> cells detected in the blood of the resistant
sheep increased significantly until 2 months p.i. when the
highest percentage of cells was detected (mean of 14%).
After 3 months p.i., both the susceptible and resistant cell
percentages decreased and a relatively constant percentage
of cells were detected in both genotype groups from 5 to 9
months p.i.

**DISCUSSION**

This study has shown differences in mononuclear cell
subsets both in lymph node cell preparations and in blood
between Suffolk sheep of susceptible and resistant PrP
genotypes following subcutaneous challenge with scrapie
inocula. A higher percentage of PrP-labelled cells was
found in the blood from sheep with a susceptible PrP
genotype at 6 months of age prior to inoculation with
scrapie. The CD21<sup>+</sup> cell population percentage was higher
in the resected prefemoral lymph nodes of susceptible
animals at 14 and 180 days p.i. Interestingly, at 14 and 180
days p.i., the CD4<sup>+</sup> population was greater in lymph nodes
from the resistant animals and the CD8<sup>+</sup>/PrP<sup>+</sup> population
was higher in the unchallenged lymph nodes, irrespective
of the PrP genotype of the sheep. In addition, the propor-
tion of CD8<sup>+</sup> and γδ T cells expressing PrP in blood from

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**Fig. 2.** Flow-cytometric analysis of target antigens: prion protein
(a), prion protein and γδ dual-labelled cells (b) and prion protein
and CD8 dual-labelled cells (c), detected in blood from
susceptible (ARQ/ARQ; ▲) and resistant sheep (ARR/ARR; ■)
found to have statistically significant patterns over time. The time
of inoculation is indicated with an arrow. Results are shown as
means ± SEM.
resistant sheep was significantly higher than in the susceptible animals in the months immediately following infection.

In general, following inoculation, the percentage of PrP+ cells detected over time was similar in both the susceptible and resistant sheep, which is in agreement with the results of Thackray et al. (2004). However, before inoculation, there were significantly more PrP-labelled cells in sheep of a susceptible genotype. These data support the findings of Herrmann et al. (2006) and Thackray et al. (2006) who reported that susceptible animals have higher numbers of cells expressing PrP and therefore a greater likelihood of these cells converting to the disease-associated PrP form, resulting in successful infection.

CD21+ B cells have been highlighted as being important in preclinical disease in deer calves orally infected with chronic wasting disease and in mice peripherally inoculated with scrapie (Frigg et al., 1999; Mabbott et al., 2001; Sigurdson et al., 2002). In our study, the CD21+ cell population, which is considered to represent B cells (Young et al., 1999), increased in genetically susceptible sheep following inoculation with scrapie brain homogenate. This increase was detected in MHC class II-labelled blood and lymph node cells, which are considered to be activated (Hopkins et al., 1989). The increase in activated B cells in the preclinical phase of scrapie may be related to the requirement of follicular dendritic cells to receive maturation stimuli from B cells in order to accumulate and possibly replicate PrPSc during peripheral scrapie infection (Mabbott & Bruce, 2002).

Genotypic differences were not found on PrP-expressing B cells following scrapie infection, contrary to the findings of Halliday et al. (2005). This apparent anomaly could be due to the different PrP antibody or the breed of sheep used. However, Thackray et al. (2006) have since reported significant differences, using mAb FH11 to detect PrP+ cells in peripheral blood and plasma, between susceptible and resistant Cheviot sheep. This was despite a lower level of PrP detection by this antibody when compared with other antibodies against PrP that detect epitopes located closer to the C terminus. The difference between the results of these two studies is most likely due to the stage of infection at which the animals were investigated, with our study assessing preclinical findings as opposed to differences at the onset of clinical symptoms. This would also explain the variance in the findings reported here with those of Davies et al. (2004), who found no significant difference in expression of lymphocyte surface markers between susceptible and resistant sheep clinically affected by scrapie.

Studies in mice and humans have shown that TSE infection results in an infiltration of CD8 and CD4 T cells into the brain and, in mice, where these phenotypes are missing the incubation period is significantly increased (Lewicki et al., 2003). In our study the number of CD4+, CD8+ and γδ T cells in blood, and to some extent in the lymph node cell preparations, varied markedly between the susceptible and resistant sheep genotypes. At 14 days p.i., the percentage of CD4+ cells in the lymph node cell preparations was higher in the group of resistant sheep and at 180 days p.i., this finding was even more pronounced. CD4+ cells, which are considered to represent T-helper/regulatory cells, are thought to be directly involved in B-cell activation in secondary lymphoid tissues. These cells, as well as macrophages and dendritic cells, are able to present antigen and release defined cytokines (Clark & Ledbetter, 1994). In our study, B cells were more abundant in the challenged lymph nodes of susceptible sheep, although a lower number of T-helper cells were detected. The B cells in the challenged lymph nodes may have been preferentially activated by migratory macrophages and dendritic cells, which are known to transport and present PrPSc, instead of T-helper cells (Huang & MacPherson, 2004).

CD8+ T cells, otherwise defined as cytotoxic T cells, are important in the successful eradication of most intracellular pathogens, and after resolution of an infection their numbers decrease rapidly (Sullivan et al., 2003). In the current study, an appreciable increase in CD8+ T cells expressing PrP was only observed in the blood shortly after inoculation and only in resistant sheep. It is tempting to suggest that the cytotoxic T cells in these animals were recognizing cells expressing the abnormal form of PrP and therefore inducing apoptosis in these cells (Kuby, 1992). The increase in the percentage of CD8+PrP+ cells may be related directly to the high proportion of γδ T cells detected, as some cells can co-express CD8 and γδ TCR epitopes on their cell surface (Roitt et al., 1998). γδ T cells are one of the first cell types to be detected at a site of inflammation and it has been suggested that they may play a major role in cell-mediated immunity (Mackay & Hein, 1991). In our study, a higher percentage of γδ T cells, which also expressed PrP, were present in the circulation of resistant sheep following inoculation. However, γδ T cells were detected at low levels in the lymph node cell preparations in both resistant and susceptible animals. This is probably due to the physiological tendency to localize in blood instead of lymph nodes (Mackay & Hein, 1991). It is therefore intriguing to question whether sheep of a resistant genotype are able to remove the scrapie agent more effectively after inoculation.

This study detected significant changes in CD21+, CD4+, CD8+ and γδ T cells in the preclinical phase of infection following administration of scrapie at a peripheral site and also showed variation in these parameters in sheep of different PrP genotypes. Our results, which support previous findings (Halliday et al., 2005; Heikenwalder et al., 2005), conflict with the traditional dogma that an immune response is not generated during scrapie infection; furthermore, they suggest that the type of immune response differs depending on the PrP genetics of the animal. However, the question that remains to be addressed is why there are differences in the immune cell subsets of infected animals, given that PrPSc is not...
recognized as a foreign antigen and a normal immune response fails to be initiated by the host following scrapie infection (Weissmann et al., 2001). Further work is required to define the very early immune responses immediately after scrapie infection. In addition, research on immune cells from different sheep breeds, using a larger number of animals, alternative routes of inoculation and different infected tissue inocula, is required to provide a greater understanding of the nature of immune system involvement in scrapie.

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