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Lymphocyte subpopulations in the blood of sheep persistently infected with Border disease virus

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SUMMARY

The surface phenotypes of peripheral blood lymphocytes in groups of lambs and adult sheep persistently infected with Border disease virus (P-I BD) were compared with those of healthy controls. The proportion and number of lymphocytes bearing surface immunoglobulin (sIg^+) and expressing class II MHC antigen (B cells) were significantly increased. A significant increase in $CD1^+$ lymphocytes was also evident. Conversely, the proportion of T lymphocytes in P-I BD lambs was reduced. A marked reduction in the proportion of circulating lymphocytes expressing class I MHC antigen was also observed. These findings were not affected by differences in the strain of the virus responsible for the persistent infection.

Keywords pestivirus T cells B cells phenotypes

INTRODUCTION

Border disease (BD) is a congenital infection of sheep caused by BD virus (BDV), a pestivirus serologically related to bovine viral diarrhoea virus (BVDV) and hog cholera virus. The infection of susceptible pregnant ewes with BDV results in viraemia and transplacental infection of the foetus. The most serious consequences follow foetal infection in the first half of gestation before the development of the foetal immune response. Foetuses which survive infection at this time may be clinically affected at birth, so-called 'hairy-shaker' lambs, and all are persistently infected (P-I) usually for life (P-I BD lambs). Such lambs are important disseminators of virus (Barlow & Patterson, 1982). The apparent tolerance in P-I BD lambs seems to be specific for the infecting virus since they are immunocompetent in the face of other antigenic stimuli (Terpstra, 1981). The same is true of cattle persistently infected with BVDV (P-I BVDV) (McClurkin *et al.*, 1984; Liess *et al.*, 1983; Steck *et al.*, 1980).

In order to elucidate the mechanisms of persistence by pestiviruses, the cells responsible for the regulation and generation of the immune response have been studied. In sheep, proportions of circulating T cells have been examined (Roeder, 1984) as well as functional aspects of the T cell response *in vitro* (Roeder, 1984; Sawyer *et al.*, 1986). In P-I BVD cattle, studies have been made of proportions of circulating leucocytes (Bolin, McClurkin & Coria, 1985; Bielefeldt-Ohmann, Ronsholt & Bloch, 1987), neutrophil function and lymphocyte blastogenesis (Roth, Bolin and Frank, 1986), and sub-populations of circulating cells containing virus antigens or cell-associated infective virus (Bielefeldt-Ohmann, Ronsholt & Bloch, 1987).

Monoclonal antibodies (MoAb) specific for sheep lymphocyte antigens have been prepared and characterized (Mackay *et al.*, 1985; Mackay, Maddox & Brandon, 1986; Maddox, Mackay & Brandon, 1985; Gogolin-Ewens *et al.*, 1985; Puri, Mackay & Brandon, 1985). The object of this study was the analysis, using these phenotypic markers, of lymphocyte subpopulations in the blood of normal lambs and lambs persistently infected with BDV.

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MATERIALS AND METHODS

Animals

P-I BD lambs. P-I BD lambs were produced by the experimental infection of susceptible oestrus-synchronized Greyface or Dorset ewes in early pregnancy with biologically cloned non-cytopathic BDV. The resulting lambs were shown to be viraemic and free of neutralising antibody both at birth and shortly before these studies were commenced. Three groups of lambs were used: groups I and II were 'hairy-shaker' lambs born to ewes infected with BDV derived from the IIB brain pool (Barlow, 1972), and group III lambs were asymptomatic at birth, their dams having been infected with the Oban strain at BDV (Bonniwell *et al.*, 1987). Lambs in group I were 18–20 months old and those in groups II and III were 8–10 months old.

Table 1. Specificity of MoAbs for sheep peripheral blood leucocytes

Cell surface antigen	Antibody clone	Specificity	Reference
CD5 (SBU-T1)	25–91	Peripheral T lymphocytes	Mackay *et al.* (1985)
CD4 (SBU-T4)	44–38	Helper T lymphocytes	Mackay, Maddox & Brandon (1986)
CD8 (SBU-T8)	38–65	Cytotoxic/suppressor T lymphocytes	Mackay, Maddox & Brandon (1986)
SBU-T19	19–19	$CD4^-$, $CD8^-$ T lymphocytes	Mackay, Maddox & Brandon (1986)
CD1 (SBU-T6)	20–27	Majority of peripheral B lymphocytes	Mackay *et al.* (1985)
MHC class I	41–19	Most somatic cells	Gogolin-Ewens *et al.* (1985)
MHC class II	28–1	B lymphocytes and activated T lymphocytes	Puri, Mackay & Brandon (1985)
SBU-LCA	1–32	All peripheral leucocytes	Maddox, Mackay & Brandon (1985)
IgG-Light chain	VPM 8	Peripheral B lymphocytes	

Table 2. Percentage representation of lymphocyte subpopulations in the peripheral blood of 18–20 month-old P-I BD sheep (Group I)

	Lymphocyte surface antigen*									
Group	CD5	CD4	CD8	SBU T19	CD1	MHC I	MHC II	SBU LCA	sIg	CD4:CD8 ratio
P-I BD sheep ($n=3$)	36·6 (31·0–41·1)	22·8 (19·6–25·2)	16·8 (15·0–18·6)	11·4 (10·1–12·8)	50·0 (41·6–65·2)	85·8 (84·5–86·8)	55·2 (47·8–65·7)	94·2 (93·6–94·8)	50·7 (41·3–63·5)	1·4 (1·3–1·5)
Healthy controls ($n=4$)	48·6† (45·8–52·0)	28·0 (24·6–31·3)	14·7 (12·6–17·1)	10·9 (9·0–12·4)	22·6† (15·6–32·4)	92·0† (87·8–95·5)	44·7 (41·5–48·4)	92·2 (85·0–97·0)	48·2 (45·2–50·5)	1·9 (1·4–2·3)

* Mean of percentage of positively staining lymphocytes; range in parentheses.
† Significantly different at $P \leqslant 0{\cdot}05$.

Table 3. Percentage representation of lymphocyte subpopulations in the peripheral blood of 8–10 month-old P-I BD lambs (group II)

	Lymphocyte surface antigen*									
Group	CD5	CD4	CD8	SBU T19	CD1	MHC I	MHC II	SBU LCA	sIg	CD4:CD8 ratio
P-I BD lambs ($n=4$)	35·8 (33·4–41·0)	19·9 (17·5–22·9)	16·1 (14·6–16·8)	10·0 (8·2–12·3)	43·0 (32·5–48·0)	85·7 (77·5–89·2)	58·6 (56·6–60·8)	94·6 (90·8–99·3)	61·6 (51·5–70·6)	1·3 (1·2–1·5)
Healthy controls ($n=3$)	60·2† (54·2–70·5)	32·0† (31·3–33·3)	18·7 (13·8–26·4)	12·3 (9·9–15·6)	23·1† (17·3–26·4)	93·5 (88·7–96·8)	47·2† (36·5–53·1)	97·6 (96·9–98·0)	43·0† (33·3–50·9)	1·9 (1·2–2·4)

* Mean of percentage of positively staining lymphocytes; range in parentheses.
† Significantly different at $P \leqslant 0{\cdot}05$.

Control lambs. Clinically normal lambs of similar age and breed to the P-I BD lambs were also studied. These were shown to be BDV-free and antibody-negative.

Antibodies

The mouse MoAbs against ovine lymphocyte antigens are described in Table 1. They were present in hybridoma supernatant fluids and used at previously determined optimal dilutions. Specific reactivity was detected using a sheep IgG anti-mouse IgG conjugated with fluorescein isothiocyanate (Sham-FITC). In the first group of animals to be examined, surface immunoglobulin-positive (sIg^+) lymphocytes (B cells) were identified using a polyvalent pig anti-sheep IgG-FITC. Subsequently, a MoAb (VPM 8) recognising ovine Ig light chain was used.

Cell Preparations

Blood samples were obtained by venepuncture into preservative-free heparin (10 i.u. ml^{-1} of blood, Evans Medical, Liverpool, UK). White blood cells (WBC) were isolated by lysis of the red blood cells using Tris-buffered ammonium chloride (Mishell and Shiigi, 1980). The cells were washed three times with phosphate-buffered saline (PBS) containing 1% bovine serum albumin, 0·5% heparin and 0·1% sodium azide (PBS-A). Leucocyte suspensions were finally adjusted to a concentration of 1×10^8 ml^{-1} using PBS-A.

Three group I sheep (18–20 months old) and four age-matched controls were each sampled on three occasions at intervals of 3–4 weeks. Four group II lambs (8–10 months old) and three age-matched controls were similarly sampled, and seven lambs comprising group III (8–10 month old 'Oban' lambs) were bled on two occasions. The arithmetic mean value and range for each animal was then calculated.

Immunofluorescent staining and flow cytometry

The method of immunofluorescent staining was essentially that described by Maddox, Mackay & Brandon (1985). Briefly, 50 μl of cell suspension (5×10^6 leucocytes) were reacted with 50 μl of appropriated MoAb at the predetermined optimal dilution for 1 h at 4°C. The cells were washed twice with PBS-A and allowed to react for a further 1 h at 4°C with Sham-FITC at a dilution of 1:50. For control purposes, a similar volume of cells was left in PBS-A at 4°C for 1 h prior to washing and addition of Sham-FITC. After washing twice with PBS-A, the cells were finally resuspended in 500 μl of fixative (PBS + 3% formalin + 2% glucose) and stored at 4°C in the dark.

The stained cells were analysed using a fluorescence-activated cell sorter (FACS IV, Becton Dickinson, Mountain View, CA). Dead cells were excluded on the basis of their size indicated by their forward light scatter. Non-lymphoid cells were excluded on the basis of their granularity indicated by their 90° light scatter. A total of 10^4 lymphocytes were then analysed for each sample, using an argon ion laser set at 400 mV, 488 nm (for excitation of FITC) and the photomultiplier voltage was set at 650 V.

Statistical analysis

Results are expressed as arithmetic means and ranges. Comparisons of means between lymphocyte sub-populations of control and P-I BD lambs were made using the Mann-Whitney rank test.

RESULTS

Group I (18–20 month-old sheep)

Proportions of lymphocyte subpopulations in the blood of these and control sheep are shown in Table 2.

A significant reduction in the proportion of circulating T lymphocytes ($CD5^+$) (37%) compared with control values (49%) was noted in P-I BD sheep. The proportions of lymphocytes staining with the B cell-reactive MoAb (anti-CD1 and anti-MHC class II) were elevated, significance being shown with the $CD1^+$ cells. This was not reflected by the values for sIg^+ cells detected with the polyvalent anti-serum. A significant reduction in the proportion of lymphocytes expressing MHC class I antigen was also recorded in P-I BD sheep. There was a slight, but not statistically significant decrease in the ratio of $CD4^+$:$CD8^+$ lymphocytes (helper T cells:cytotoxic-suppressor T cells) in P-I BD sheep.

Group II (8–10 month-old lambs)

The proportion of T lymphocytes ($CD5^+$) in P-I BD lambs (36%) in this group was significantly reduced when compared with control values (60%) (Table 3). The reduction in $CD5^+$ cells was reflected by a statistically significant increase in the proportion of B cells (62% in P-I BD lambs compared with 43% in healthy lambs) detected with a MoAb directed against immunoglobulin light chain (VPM 8). The proportion of $CD1^+$ cells was also significantly increased (43% in P-I BD and 25% in control lambs), as was the proportion of MHC class II-bearing lymphocytes (59% in P-I BD lambs; 47% in healthy lambs). The reduced ratio of $CD4^+$:$CD8^+$ (helper:cytotoxic-suppressor) lymphocytes in P-I BD lambs was not significant although the proportion of $CD4^+$ cells (20%) was markedly lower than the control values (32%). There was also a reduced proportion of cells expressing MHC class I antigen in these younger P-I BD lambs.

To evaluate the contribution of absolute numbers of T and B lymphocytes to the above results, total WBC and differential cell counts were made. Total cell counts in P-I BD lambs were significantly raised ($P \leqslant 0{\cdot}05$), while differential counts were similar in affected and control lambs. Consequently, the number of lymphocytes/ml^{-1} of blood was significantly elevated in the P-I BD group ($P \leqslant 0{\cdot}05$).

When expressed as absolute numbers of cells ($\times 10^5$ ml^{-1} of blood), the content of $CD5^+$ cells was similar in both infected and control lambs (Table 4), but the numbers of cells expressing sIg (VPM8), MHC class II and CD1 were all significantly increased in P-I BD lambs.

Group III (8–10 month-old 'Oban' lambs)

Sub-populations of PBL from this group of seven lambs were compared with those from the previous controls (8–10 month-old lambs, Table 3).

The proportions of $CD5^+$ and $CD4^+$ cells were significantly reduced in the 'Oban' P-I BD lambs compared with those of the controls (Table 5). In contrast, surface parameters associated with B cells, including sIg (60%) and CD1 (58%), were significantly increased over control values (43% and 23% respectively). Lymphocytes expressing MHC class II antigen (60%) were elevated over control proportions (47%), although significance was not shown.

When the results from this group were expressed as absolute numbers, the content of circulating $CD5^+$ lymphocytes was similar to that of the controls (Table 6), whereas numbers of cells expressing sIg, MHC class II and CD1 were significantly elevated in the P-I BD animals.

Comparisons between the percentages of lymphocyte sub-populations from group III P-I BD lambs and those from group II P-I BD lambs indicated no significant diffferences between the two affected groups.

DISCUSSION

The cellular compartment of the immunological deficiency in P-I BD sheep has not previously been characterized. The present results demonstrate perturbation of the peripheral blood lym-

Table 4. Absolute numbers of lymphocyte subpopulations in the peripheral blood of 8–10 month-old P-I BD lambs (group II)

Group	No. of lymphs (10^5/ml)	Lymphocyte surface antigen* CD5	CD4	CD8	SBU T19	CD1	MHC I	MHC II	SBU LCA	sIg	CD4:CD8 ratio
P-I BD lambs ($n=4$)	65·7 (55·0–74·0)	23·0 (22·3–25·1)	12·7 (12·0–13·3)	10·1 (7·9–12·0)	6·7 (5·0–8·0)	27·7 (24·3–30·7)	53·7 (41·1–63·6)	39·0 (32·6–43·2)	62·6 (52·3–73·5)	40·3 (35·6–45·3)	1·3 (1·2–1·5)
Healthy Controls ($n=3$)	46·4 (42·0–52·0)	29·0 (23·0–35·9)	15·2† (14·0–16·0)	8·9 (6·6–13·7)	5·5 (3·6–6·4)	11·3† (9·2–13·5)	44·0 (40·1–49·4)	20·4† (18·6–21·6)	45·4 (40·9–50·1)	17·8† (17·0–18·7)	1·9 (1·2–2·4)

* Mean of absolute number ($\times 10^5$ ml^{-1}) of positively staining lymphocytes; range in parentheses.
† Significantly different at $P \leqslant 0{\cdot}05$.

Table 5. Percentage representation of lymphocyte subpopulations in the peripheral blood of 8–10 month-old P-I BD (Oban strain) lambs (group III)

Group	Lymphocyte surface antigen* CD5	CD4	CD8	SBU T19	CD1	MHC I	MHC II	SBU LCA	sIg	CD4:CD8 ratio
P-I BD 'Oban' lambs ($n=7$)	38·8 (28·1–49·3)	22·2 (17·1–29·9)	13·4 (10·0–15·5)	13·6 (10·5–15·4)	58·4 (39·4–71·3)	72·1 (57·6–84·6)	60·1 (49·9–71·7)	96·4 (93·1–98·7)	59·7 (47·3–67·9)	1·7 (1·4–2·9)
Healthy controls ($n=3$)	60·2† (54·2–70·5)	32·0† (31·3–33·3)	18·7 (13·8–26·4)	12·3 (9·9–15·6)	23·1† (17·3–26·4)	93·5† (88·7–96·8)	47·2 (36·5–53·1)	97·6 (96·9–98·0)	43·0† (33·3–50·9)	1·9 (1·2–2·4)

* Mean of percentage of positively staining lymphocytes; range in parentheses.
† Significantly different at $P \leqslant 0{\cdot}05$.

Table 6. Absolute numbers of lymphocyte subpopulations in the peripheral blood of 8–10 month-old P-I BD (Oban strain) lambs (group III)

Group	No. of lymphs (10^5/ml)	Lymphocyte surface antigen* CD5	CD4	CD8	SBU T19	CD1	MHC I	MHC II	SBU LCA	sIg	CD4:CD8 ratio
P-I BD lambs ($n=7$)	88·0 (71·2–138·8)	35·2 (21·1–53·5)	20·9 (12·7–36·3)	12·1 (6·6–21·0)	12·3 (7·7–17·7)	51·9 (34·3–95·7)	63·0 (41·9–81·2)	53·2 (40·3–92·2)	82·5 (66·5–125·3)	52·8 (38·5–95·8)	1·7 (1·4–2·1)
Healthy controls ($n=3$)	46·4† (42·0–51·0)	29·0 (23·0–35·9)	15·2 (14·0–16·0)	8·9 (6·6–13·7)	5·5† (3·6–6·4)	11·3† (9·2–13·5)	44·0† (40·1–49·4)	20·4† (18·6–21·6)	45·4† (40·9–50·1)	17·8† (17·0–18·7)	1·9 (1·2–2·4)

* Mean of absolute number ($\times 10^5$ ml^{-1}) of positively staining lymphocytes; range in parentheses.
† Significantly different at $P \leqslant 0{\cdot}05$.

phocyte subpopulations in such animals. Altered proportions of T and B cells were detected in all P-I BD animals and the underlying trends were of B cell hyperplasia, increases in the B cell-associated surface antigens and reduced proportions of cells expressing MHC class I antigens.

Using peanut agglutinin, a putative T cell marker, Roeder (1984) noted reduced numbers of circulating peanut agglutinin-reactive cells in P-I BD lambs. The present results indicate that reduced proportions of the $CD5^+$ (T cells) and, to a lesser extent the $CD4^+$ (helper) phenotype, in association with B cell hyperplasia are the principal changes associated with infection. Similar findings have been reported in cattle persistently infected with the related BVD pestivirus (Bielefeldt-Ohmann, Ronsholt & Bloch, 1987) where the proportion of $CD5^+$ (T) cells was reduced and B cells were elevated in peripheral blood.

Two important properties in the establishment and maintenance of viral persistence are low or no cytopathogenicity, and ability to infect lymphocytes and macrophages (Mims, 1974). It is significant, therefore, that P-I BD lambs can remain clinically unaffected and that nearly all BDV isolates are non-cytopathic *in vitro* (Hadjisavvas *et al.*, 1975; Harkness *et al.*, 1977; Terpstra, 1978). Furthermore, the closely related BVDV replicates *in vitro* in both lymphocytes and macrophages, and replication is enhanced by stimulation with phytohaemagglutinin (Truitt & Schechmeister, 1973). BVD viral antigen is strongly associated with cells of the mononuclear phagocyte system in experimentally inoculated foetuses (Bielefeldt-Ohmann *et al.*, 1982) and has also been detected immunohistochemically in association with 5–36% of peripheral blood mononuclear cells from P-I cattle (Bielefeldt-Ohmann, Ronsholt & Bloch, 1987). Viral infection and replication were demonstrated in T lymphocytes, null cells, monocytes and, to a lesser extent, B lymphocytes (Bielefeldt-Ohmann, Ronsholt & Bloch, 1987; Bolin, Sacks & Crowder, 1987). It is possible that the pathogenesis of BD is similar, and future work may determine whether the virus is associated with any particular lymphocyte subset.

The functional significance of the changes in PBL phenotypes occurring in P-I BD sheep is not known. Normal Ig values and immune responsiveness against unrelated antigens, including super-infecting pestiviruses, are both reported to occur in affected sheep and cattle (Coria and McClurkin, 1978; Terpstra, 1981), indicating that tolerance is directed against specific viral antigens in both species (Gardiner, Nettleton & Barlow, 1983; Steck *et al.*, 1980; Liess *et al.*, 1983). Nevertheless, lymphocytes from P-I BD lambs and from P-I BVD cattle exhibit reduced *in vitro* responses to mitogens (Johnson & Muscoplat, 1973; Roeder, 1984; Roth, Bolin & Frank, 1986; Sawyer *et al.*, 1986). While the proportion of $CD4^+$ cells was reduced in blood there was no commensurate increase in $CD8^+$ cells in P-I BD lambs. Perhaps of more functional significance were the reduced percentages of lymphocytes expressing MHC class I antigens. The role of class I antigens in directing cytotoxic effector cells against virus-infected targets is well established (Zinkernagel & Doherty, 1974) and there is a suggestion that reduced expression of this antigen may enable adenoviruses to evade immune surveillance (Andersson *et al.*, 1985; Hayashi *et al.*, 1985) or lead to reduced lysis of Herpes simplex-infected cells (Jennings *et al.*, 1985).

Although the $CD1^+$ phenotype is contained within the sIg^+ subpopulation (Mackay *et al.*, 1985), the identity and relevance of this population in the peripheral blood of sheep is uncertain. Nevertheless, it is interesting that this sub-population is significantly elevated in P-I BD lambs as are the other B-cell markers.

The effects on the immune system of the changes described in this report are not known. However, the application of a biochemically characterized panel of MoAb has revealed a number of subtle changes present in P-I BD lambs irrespective of age, clinical status, or strain of virus. These findings, therefore, provide a basis for analysis of the immune perturbation associated with BDV persistence.

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