

Cell phenotypes in the efferent lymph of sheep persistently infected with Border disease virus

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SUMMARY

The prefemoral efferent lymphatics of sheep persistently infected (PI) with Border disease virus (BDV) were cannulated in order to study the effects of the virus on cells of the immune system. Efferent lymphocytes recovered from PI sheep were phenotyped using a panel of monoclonal antibodies (MoAb) specific for ovine cell-surface markers and compared to lymphocytes recovered from normal, healthy controls. PI sheep had an increased percentage of cells expressing the T cell-associated molecules CD5, CD4, CD8 and T19, also an increase in cells expressing CD1 and a population of cells expressing low levels of the T19 molecule which was not present in control sheep. The lymphocytes were examined for the presence of BDV using virus-specific MoAb. On average 8.5% of the efferent lymphocytes from PI sheep carried virus antigen. BDV antigen was also found in the mononuclear cells and connective tissue of lymph nodes indicating widespread virus dissemination within the lymphoid system of PI sheep.

Keywords Border disease virus persistence lymphatic cannulation

INTRODUCTION

Border disease is a viral infection of sheep which, in pregnant ewes, can cause abortion or the birth of lambs with abnormalities of the nervous and skeletal systems [1]. The causative agent Border disease virus (BDV), is an ovine pestivirus serologically related to bovine virus diarrhoea virus (BVDV) and hog cholera virus (HCV) [2]. Fetal infection during early pregnancy results in the birth of lambs which are persistently infected (PI) with BDV. Such animals continuously excrete virus and appear to be immunotolerant to BDV, thereby acting as a source of infection for other sheep [3]. Subsequent studies have revealed that PI sheep excrete only the noncytopathic (NCP) biotype of BDV [4]. Experimental inoculation of PI sheep with a cytopathic (CP) biotype of BDV can result in the development of a fatal mucosal disease (MD)-like syndrome characterized by lymphoproliferation, mucosal discharge and intractable diarrhoea [5]. In these cases and in cases of spontaneous MD-like syndrome, CP virus can be recovered from PI sheep [4].

In order to study this apparent immunotolerance to BDV we are investigating how the virus may affect the immune system of PI sheep. Previously, PI sheep have been shown to have abnormal proportions of circulating lymphocytes in peripheral blood, reflecting a B cell hyperplasia [6]. To extend those studies the cell phenotypes in efferent lymph in PI animals are being analysed and the cells examined for the presence of virus. We report here on the phenotype profiles of efferent lymph cells in PI sheep and demonstrate the presence of BDV in these cells and in lymph node tissue.

MATERIALS AND METHODS

Animals

PI sheep were the progeny of Dorset dams experimentally infected in early pregnancy with the Oban strain of BDV isolated from a natural outbreak of BD in the West of Scotland [7]. They were viraemic and free of neutralizing antibody both at birth and also before cannulation. The efferent duct draining the prefemoral lymph node was cannulated as described by Hall [8]. Lymph was collected quantitatively into sterile, siliconized plastic bottles containing 100 i.u. heparin. Sheep were housed in metabolism crates and given hay and water *ad libitum*.

Lymph nodes

The right and left prefemoral lymph nodes were excised from all four sheep 2–4 months after cannulation. Fragments of nodes were either fixed in Bakers Formol Calcium for histology or snap-frozen in OCT compound (Miles Laboratories) by immersion in liquid nitrogen then stored at -80°C for immunocytochemistry.

Immunocytochemistry

Efferent lymph (EL) cells, cytocentrifuged onto glass slides and air dried, were fixed in ice-cold acetone for 10 min and stored at -20°C . The cytospin slides were brought to room temperature and washed with buffer consisting of 0.5% w/v ovalbumin (Sigma) and 0.05% Tween 80 (Sigma) dissolved in PBS. This wash buffer was used throughout the immunocytochemical procedure. Fixed cells were incubated for 2 h with a pool of MoAbs which react with BDV. This pool consisted of equal volumes of tissue culture supernatant of the MoAb VPM20, VPM21, VPM22, VPM26 and VPM49 [9]. VPM20, 26 and 49

are IgG2a isotype, VPM21 is IgG1 and VPM22 is IgG2b. A rotavirus-specific MoAb of IgG1 isotype was used as a negative control (kindly supplied by Iris Campbell, Moredun Research Institute). After washing, the cells were reacted with a sheep anti-mouse horseradish peroxidase conjugate for 1 h and diaminobenzidine substrate (Sigma) was added for 10 min. The slides were then washed in running tap water and the cells counterstained with haematoxylin (Gurr), dehydrated and mounted. The number of virus-infected, peroxidase-labelled cells was determined by examining 500 cells per slide and counting the number of cells with positive staining.

Frozen sections of lymph node were air-dried for 1 h then fixed and stored as described for cytopins. Staining was performed as described for cytopins but with the inclusion of a step to block endogenous peroxidase activity. For this, the slides were incubated in 3% H₂O₂/PBS for 10 min after the first antibody and before the addition of conjugate.

Phenotypic analysis of efferent lymph cells

An indirect immunofluorescence test was used. Washed efferent lymphocytes (2×10^6) were incubated for 60 min at 4°C with 50 µl of a 1/1000 dilution of MoAb ascitic fluid in a 0.1% solution of bovine serum albumin (BSA) in PBS containing 0.01 M sodium azide. The antibodies used (purchased from Dr M. Brandon, Melbourne) are detailed in Table 1. Their characterization and cellular reactivities have been published [10-14]. After the incubation with primary antibody the cells were washed and incubated for a further 60 min in the appropriate dilution of FITC-conjugated sheep anti-mouse immunoglobulin (Ig) in PBS/BSA/azide. The percentage of positive cells was determined by reference to the 1/500 normal mouse serum negative control.

After the final wash the cells were resuspended in PBS/BSA/azide and fixed by the addition of an equal volume of fresh 1% paraformaldehyde. Flow cytometry was performed using a Becton Dickenson Facscan (Mountain View, CA) with linear amplification for forward (FSC) and side scatter (SSC) and logarithmic amplification for FITC green fluorescence (FL-1). Small lymphocytes were distinguished on the basis of FSC/SSC profile.

Table 1. Cell phenotypes in the efferent lymph of sheep persistently infected (PI) with Border disease virus

Antibody	Specificity	Percentage positive cells in	
		PI sheep*	Control sheep*
SBU-T1	CD5	84 (3.3)	74 (2.6)†
SBU-T4	CD4	49 (1.6)	39 (2.5)**
SBU-T8	CD8	25 (2.1)	16 (1.5)**
SBU-T19	CD4 ⁻ CD8 ⁻ CD5 ⁺ cells	26 (1.7)	10 (1.5)***
SBU-T6	CD1	22 (3.0)	8 (1.3)***
SBU-II(28-1)	MHC class II	34 (2.2)	36 (2.1)
SBU-I (41-19)	MHC class I	94 (2.1)	99 (0.5)
SBU-LCA	CD45	97 (1.6)	99 (0.7)
VPM 8	Ig Light chain	19 (3.4)	26 (2.3)
VPM22	BDV	1.8 (0.7)	1.5 (0.5)

* $n=4$, Standard error of the mean in parentheses. † $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

Facscan analysis of efferent lymph cells for the presence of BDV surface antigen

Efferent lymph cells, prepared as above, were labelled with the anti-BDV MoAb VPM22 followed by a FITC-conjugated sheep anti-mouse Ig. The various washes and the analysis were performed as described in the previous section.

Statistical analysis

Results are expressed as arithmetic means and s.d. Comparison of means between PI and control sheep were performed using the Student *t*-test.

RESULTS

Flow cytometry revealed that efferent lymphocytes recovered from PI sheep have a different phenotype profile compared to age-matched uninfected controls. There were significant increases in the percentage of cells expressing the T cell markers CD5, CD4, CD8 and T19 (Table 1). A notable difference between PI and control sheep was in the expression of the T19 molecule. The percentages of cells expressing high levels of T19

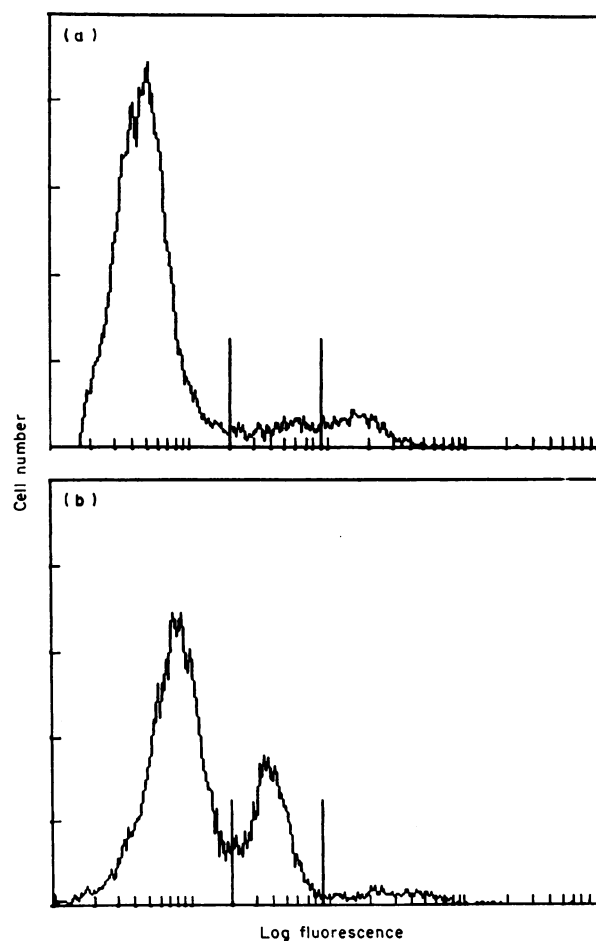


Fig. 1. Facscan profile of T19-positive efferent lymphocytes. Cell number plotted against log fluorescence. A. Uninfected, control sheep. B. Persistently infected (PI) sheep. The percentage of efferent lymphocytes expressing low levels of T19 is greatly increased in PI sheep. The percentage of cells expressing high levels of T19 is similar in both groups.

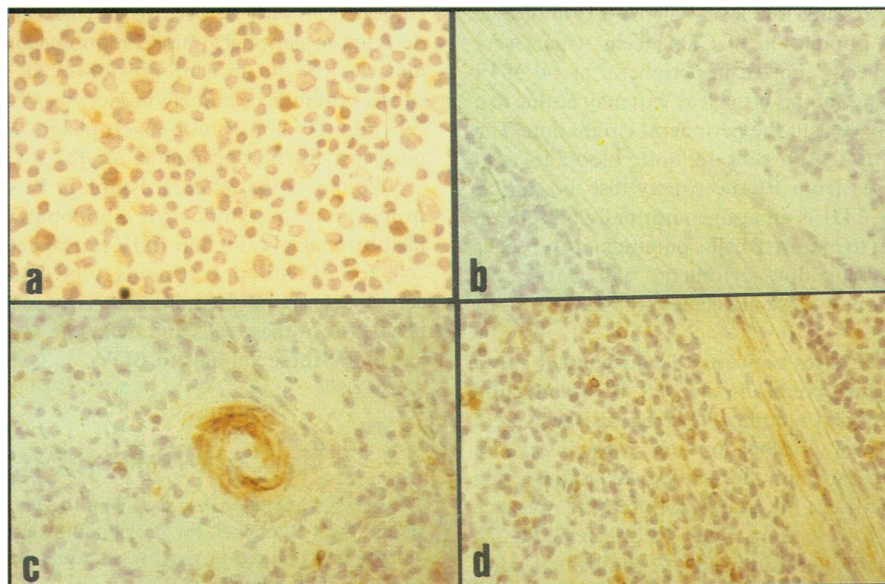


Fig. 2. Cytospin preparation of efferent lymph cells from a PI sheep reacted with anti-BD MoAb. Approximately 10% of the cells stain positive for virus antigen. All cells are lymphocytes. $\times 250$. (b) Cryostat section of lymph node from a control sheep reacted with anti-BD MoAb showing negative staining pattern. $\times 250$. (c) and (d). Cryostat sections of prefemoral lymph node from a PI sheep reacted with anti-BD MoAbs. (c) Viral antigen concentrated in the wall of an arteriole. (d) Viral antigen present in the mononuclear cells and in the fibrous structure of the trabeculum. $\times 250$.

were similar in both groups of sheep (Fig. 1). However, in PI sheep there was a population of cells expressing low levels of the molecule not present in control sheep. The total percentage of cells expressing the T cell markers CD4, CD8 and T19 in control sheep (65%) was less than the percentage of cells expressing CD5 (74%). In PI sheep, the combined total of CD4, CD8 and T19 (100%) greatly exceeded CD5 expression (84%). One other striking phenotypic difference between cells from the two groups was the increased percentage of cells expressing CD1 in PI sheep (Table 1).

Virus antigen could not be detected on the surface of the efferent lymphocytes using the MoAb VPM22 (Table 1). However, virus antigen was demonstrated in acetone-fixed, cytocentrifuge preparations of the cells using virus-specific MoAb. Immunoperoxidase staining revealed that the prefemoral efferent lymphocytes carried virus antigen (Fig. 2a). Controls confirmed the specificity of this reaction (not shown). The percentages of efferent lymphocytes which stained positive for viral antigen from the four sheep were 14.6%, 7.8%, 6.4% and 5.0%. The average of these four observations is 8.5%.

BDV antigen was not detected in lymph node sections from uninfected animals reacted with the BDV MoAb (Fig. 2b) or in lymph node sections from PI animals reacted with the control MoAb (not shown). However, virus antigen was found extensively in lymph nodes from PI sheep reacted with BDV-specific MoAb. Staining was not confined to any particular area, being present in mononuclear cells in the cortex and medulla as well as in the cells which constitute the fibrous structure of the node itself (Fig. 2c, d). Examination of formol-fixed sections stained with haematoxylin and eosin revealed no gross histopathological changes in the nodes. They had the appearance of normal, resting lymph nodes (not shown).

DISCUSSION

Phenotypic abnormalities in lymphocyte subpopulations in the peripheral blood of PI sheep have been reported previously [6]. Those abnormalities consisted of a B cell hyperplasia, an increase in the percentage of cells expressing CD1 and a decrease in the percentage of cells expressing CD5. However, an increase in CD8 cells in the peripheral blood of PI sheep also has been reported [15]. In the efferent lymph of PI sheep we have found an increase in the percentage of cells expressing the T cell associated molecules CD5, CD4, CD8 and T19. We have also found an increase in the percentage of cells expressing CD1 (Table 1). These different observations may be due to the fact that efferent lymph contains an almost pure population of lymphocytes and therefore has a very different cellular composition from blood [16]. In the efferent lymph of PI sheep the combined percentage of cells expressing CD4, CD8 and T19 greatly exceeded the percentage of cells expressing CD5 (Table 1). This is also true for peripheral blood cells from PI sheep [6]. This is an unusual finding, since the MoAb recognizing CD4, CD8 and T19 have been shown to react with mutually exclusive T cell subpopulations as defined by CD5 expression in sheep [14]. This implies the co-expression of some combination of CD4, CD8 or T19 on T cells in the periphery in PI sheep. Co-expression of all combinations of these markers on sheep efferent lymphocytes has been described previously, but only following *in vitro* mitogenic stimulation of the cells and not on freshly recovered cells [17].

The expression of the T19 molecule on cells from PI sheep differed significantly from that on cells from normal control sheep. The T19 molecule is a surface protein on T cells expressing the $\gamma\delta$ receptor and is unique to ruminants [18]. In efferent lymphocytes from PI sheep we have found a distinct

population of cells expressing low levels of the T19 molecule (Fig. 1). An increased population of CD5⁺ cells which were CD4⁻ and CD8⁻ has been reported in peripheral blood of PI sheep [15]. The significance of this is unclear, but may be due to a population of immature cells in the peripheral circulation. The increased percentage of CD1⁺ cells in both blood [6] and efferent lymph (Table 1) from PI sheep may also be due to immature T cells, since CD1 is an antigen normally present on thymocytes (in addition to mature B cells) but not mature T cells in sheep [19]. Two-colour flow cytometry will clarify the question of co-expression of these molecules on T cells from PI sheep.

The phenotypic differences between PI and control sheep which we have identified could be due to BDV infecting lymphocytes *in vivo*. Efferent lymphocytes from PI sheep were analysed for the presence of virus by immunocytochemistry and by FACS analysis. Previous immunohistological studies using anti-BDV polyclonal antisera have demonstrated the presence of virus in cryostat tissue sections from PI sheep [3,20–22]. Those studies were performed using a FITC-conjugated polyclonal sheep anti-BD serum and showed the virus to be present in many tissues. Such a serum can be used only as a direct conjugate since secondary anti-sheep antibodies would also react with endogenous immunoglobulins. Therefore, the association of BDV with leucocytes from PI sheep has been demonstrated only indirectly, by co-cultivation of peripheral blood mononuclear cells from PI animals with virus-permissive cells which were then subsequently stained for virus [3,23,24]. The method which we describe in this report to demonstrate BDV in lymphocytes has advantages over those previously published techniques, both in sensitivity and specificity. The sensitivity of the technique abrogates the need for amplification of viral antigen by replication in secondary cells and the direct visualization of BDV antigen in lymphocytes by virus-specific MoAb allows enumeration of infected cells, thus providing an insight into dissemination of virus by recirculating cells in PI sheep. Infectious BDV was found to be associated with the efferent lymphocytes from two of these PI sheep by co-cultivation with virus-permissive ovine fibroblasts (results not shown). The frequency of cells carrying infectious virus was not determined.

The MoAbs used in these studies all react with a 120 kD polypeptide in NCP BDV-infected cell lysates [9]. This polypeptide appears to be non-structural and highly conserved among pestiviruses [25]. Therefore, the MoAbs do not recognize virus particles but identify cells which carry viral antigen as a result of viral replication. This may have a particular bearing on the ability of these MoAbs to identify virus-infected lymphocytes by FACS analysis. We have been unable to identify infected cells by FACS analysis using VPM 22, indicating that the non-structural polypeptide of BDV is not expressed on the surface of virus-infected cells. Recently, pestivirus antigen has been detected by flow cytometry in peripheral blood cells from cattle persistently infected with BVDV but only after the cell membranes were permeabilized to allow the antibody access to the cytoplasm [26].

We have identified lymphocytes as a source of virus in PI sheep but have not yet been able to identify the particular subsets which may be infected. However, considerable progress has been made in the identification of peripheral blood cells in cattle which harbour BVDV, a pestivirus closely related to BDV. In experiments using a polyclonal antiserum to BVDV in

conjunction with MoAbs to bovine cell subsets, BVDV was detected in peripheral blood mononuclear cells from PI cattle and was present in a proportion of all the mononuclear cell subsets examined, namely monocytes, T cells, B cells and null cells (non-T, non-B, non-macrophage), with T cells being the most heavily infected population [27,28].

We report that efferent lymph in PI sheep contains altered proportions of lymphocyte subpopulations compared with healthy controls and that BDV can be detected in some of these cells from PI sheep. It remains to be determined which specific cell subsets are infected, how the virus may affect the functional behaviour of these cells and their role, if any, in the immunotolerance of these animals to BDV.

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