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Phenotypic analysis of cells in bronchoalveolar lavage fluid and peripheral blood of maedi visna-infected sheep

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SUMMARY

A phenotypic analysis of bronchoalveolar lavage fluid (BALF) and peripheral blood (PB) cells in maedi visna virus (MVV)-infected sheep has been performed. The differential cell count in BALF from MVV-infected animals was characterized by a significant increase ($P < 0.05$) in lymphocytes and neutrophils. Lymphocyte phenotyping in BALF from MVV-infected sheep showed a significant decrease ($P < 0.05$) of $CD4^+$ cells, a significant increase ($P < 0.05$) of $CD8^+$ cells and a significant inversion ($P < 0.001$) of the $CD4^+/CD8^+$ ratio. $CD5^+$ lymphocytes were also significantly decreased ($P < 0.05$). $\gamma\delta$ T cells and B cells did not differ significantly when compared with the controls. No correlation was observed between BALF and PB lymphocyte phenotypes. BALF macrophages from MVV-infected animals showed increased MHC class II expression and BALF lymphocytes from the same animals demonstrated up-regulation of LFA-1 and LFA-3 expression. These findings and their relationship with lentiviral pathogenesis are discussed.

Keywords maedi lentivirus bronchoalveolar lavage cell phenotype sheep

INTRODUCTION

Maedi visna virus (MVV) is a lentivirus responsible for an ovine multisystemic disease, a chronic progressive interstitial pneumonia being one of its main clinical manifestations [1,2]. Other manifestations are meningoencephalitis, indurative mastitis and arthritis [3–5]. The virus causes persistent infection of the monocyte/macrophage cell lineage leading to chronic inflammatory lesions [6,7]. In the interstitial lung disease, lesions consist of interstitial mononuclear cell infiltration, lymphoid tissue and smooth muscle hyperplasia [8]. The study of the interstitial lung diseases both in humans and animals is a vast research field, the etiology of most of these diseases still being unknown. The study of the immune response in the lungs during the course of MVV infection might provide useful information about the pathogenesis of the lentiviral-induced interstitial pneumonias, including the lymphocytic pneumonia observed in AIDS [9]. This study reports the phenotypic alterations induced by natural MVV infection in cells recovered by bronchoalveolar lavage (BAL) and the absence of changes in peripheral blood lymphocytes (PBL).

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MATERIAL AND METHODS

Animals, bronchoalveolar lavage technique and sample processing

Twelve adult Texel ewes, seropositive for MVV by the agar gel immunodiffusion test (AGID) [10] and 13 breed, sex, and age-matched seronegative control ewes were used. The seropositive animals were selected from a naturally infected flock [11] and represented different stages of clinical respiratory disease severity, as assessed by pulmonary function testing (Collie *et al.*, Am J Vet Res, manuscript accepted). The control animals were purchased from an accredited MVV-free Texel flock. Animals were kept in separate buildings but under similar management conditions.

Animals were anaesthetized with intravenous thiopentone sodium (20 mg/kg) (Intraval Sodium; May & Baker Ltd), intubated with 9·5–10·5 mm cuffed endotracheal tubes and placed in sternal recumbency. A fibreoptic bronchoscope (American Optical) was introduced through the endotracheal tube and wedged in the lobar bronchus of the diaphragmatic lobe. Two hundred millilitres of sterile saline solution, divided into four aliquots of 50 ml, were infused through the lumen of the bronchoscope. A gentle aspiration of the bronchoalveolar lavage fluid (BALF) was carried out after each of the infusions and the four recoveries were pooled together in precooled plastic containers. Peripheral blood (PB) samples were taken from each animal by jugular venepuncture and collected into EDTA tubes.

BALF was filtered through a loose cotton gauze and the recovered volume measured, the normal recovery being between

40% and 60%. Cell pellets were harvested by a 10 min centrifugation at 800 *g* and resuspended in sterile PBS. A total BALF cell count was performed by using an automatic counter (Serono, Baker Diagnostics). Viability of the cells, determined by trypan blue dye exclusion, was always higher than 90%. Finally, cytocentrifuge smears were prepared (Cystospin 3, Shandon) and stained with Leishmann's dye for differential cell counting. In blood, erythrocytes were lysed with NH_4Cl buffer [12] and a total and differential leucocyte count was performed.

Immunofluorescent labelling technique

The reactivity of the MoAbs used in this study was: Leucocyte Common Antigen (LCA, CD45 analogue) (VPM 18), CD4 (17D.13), CD8 (SBU-T8), $\gamma\delta$ TCR (86D), CD5 (ST1), IgG light chain for B cells (VPM 8), a Pan MHC class II reagent (VPM 36+VPM 38), an MHC class II-DR (VPM 37), an MHC class II-DQ (VPM 41) and the Lymphocyte Function-Associated Antigen-1 (LFA-1) (F10-150-39) and 3 (LFA-3) (L180/1) [13–21]. All MoAbs were used on lymphocytes and macrophages recovered from BALF, while only the first seven MoAbs were used for analysis of lymphocytes in PB. MoAbs were used as hybridoma supernatant fluids at previously determined optimal dilutions.

The procedure was essentially the same for both BALF and PB samples. Cells ($1{\cdot}5 \times 10^5$) were incubated with 50 μl of primary MoAbs for 45 min at 4°C. Cells were then washed three times with PBA (PBS+2% bovine serum albumin +0·1% sodium azide) and incubated with 50 μl of a 1:100 dilution FITC-conjugated rabbit $F(ab')_2$ anti-mouse immunoglobulin (whole molecule) (Dako) for 30 min at 4°C in the dark. Three further washes with PBA were performed and cells were finally fixed in a 1% paraformaldehyde solution (pH 7·2) and kept at 4°C until analysed within 24 h.

Flow cytometry, data analysis and statistics

Samples were analysed on a FACScan flow cytometer (Becton Dickinson). Ten thousand individual cells from each sample were analysed by setting separate live gates around the lymphocyte or macrophage populations respectively, being distinguished by their FSC/SSC profiles. The macrophage autofluorescence was corrected by setting an appropriate fluorescence threshold value for each animal by using control cells stained without the addition of MoAb. Consort 30 and Lysis software packages were used to analyse data. The results were expressed either as the percentage of cells positively stained or as the value of the mode channel number of fluorescence intensity. All the sample comparisons between MVV-infected and control animals were made using the Mann–Whitney non-parametric rank test.

RESULTS

Total and differential BALF cell count

BALF from MVV-infected animals presented a slightly increased cellularity when compared with controls (MVV ($n=12$) $= 3{\cdot}9 \times 10^6$ (range 0·2–6·3); control ($n=13$) $= 2{\cdot}5 \times 10^6$ (range 1·6–6·9) (values expressed as median of cells per millilitre)) although this difference was not significant. MVV-infected animals also had significantly increased ($P<0{\cdot}05$) lymphocytes and neutrophils and significantly decreased macrophages ($P<0{\cdot}05$) (Table 1). However, absolute number showed a significant increase of lymphocytes ($P<0{\cdot}001$), neutrophils ($P<0{\cdot}001$) and macrophages ($P<0{\cdot}05$) (data not shown).

Lymphocyte phenotyping in BALF and PB (Table 2)

In BALF of MVV-infected animals, the most noticeable changes were a significant decrease ($P<0{\cdot}05$) of $CD4^+$ lymphocytes and a significant increase ($P<0{\cdot}05$) of $CD8^+$ T lymphocytes ratios, leading to a significant inversion ($P<0{\cdot}001$) of the $CD4^+/CD8^+$ ratio when compared with controls. A significant decrease ($P<0{\cdot}05$) of $CD5^+$ lymphocytes was also observed. The percentage of $\gamma\delta$ T cells in BALF did not show statistical variations and B cells in BALF of MVV-infected animals were increased, although this difference was not statistically significant. In PB, no significant differences in lymphocyte subsets between control and MVV-infected sheep were found for any of the reagents used (data not shown). The $CD4^+/CD8^+$ ratio in

Table 1. Differential cell count in bronchoalveolar lavage fluid (BALF) from control and maedi visna virus (MVV)-infected animals

	Control ($n=9$)	Maedi ($n=12$)
Neutrophils	1·0 (0·0–3·0)*	5·5† (1·0–30·0)
Lymphocytes	5·0 (3·0–20·0)	13·0† (6·0–57·0)
Macrophages	86·0 (79·0–91·0)	69·5† (37·0–91·0)
Eosinophils	4·0 (0·0–11·0)	1·0 (0·0–9·0)
Mast cells	1·0 (0·0–2·0)	0·5 (0·0–2·0)

* Results are expressed as median of percentages with ranges in parentheses.

† Significantly different from controls at $P<0{\cdot}05$ (Mann–Whitney non-parametric rank test).

Table 2. Lymphocyte subsets in bronchoalveolar lavage fluid (BALF) from control and maedi visna virus (MVV)-infected animals

	CD45	CD4	CD8	$\gamma\delta$TCR	CD5	B cells	CD4/CD8 ratio
Control	86·9*	54·6	18·8	8·3	53·9	21·1	3·0
($n=13$)	(68·4–96·7)	(42·3–67·1)	(8·5–30·4)	(3·8–19·6)	(40·6–66·9)	(13·1–51·1)	(1·8–5·3)
Maedi	89·6	33·4†	29·0†	5·4	32·6†	33·1	0·9‡
($n=12$)	(73·3–98·9)	(10·6–67·1)	(2·9–68·3)	(1·8–25·8)	(10·7–57·6)	(11·9–92·6)	(0·3–4·0)

* Results are expressed as median of percentages of positively labelled cells in lymphocyte gate with ranges in parentheses.

† Significantly different from controls at $P<0{\cdot}05$ (Mann–Whitney non-parametric rank test).

‡ Significantly different from controls at $P<0{\cdot}001$ (Mann–Whitney non-parametric rank test).

Table 3. MHC class II expression on bronchoalveolar lavage fluid (BALF) macrophages from control and maedi visna virus (MVV)-infected animals

	Pan class II	VPM 37 (DR)	VPM 41 (DQ)
Control	57·1*	37·1	43·6
($n=13$)	(10·5–233·7)	(5·87–51·26)	(9·4–195·1)
Maedi	251·2†	98·2‡	175·0†
($n=12$)	(41·3–827·3)	(33·2–242·3)	(39·8–270·0)

* Results are expressed as median of the value of mode channel number of fluorescence intensity with ranges in parentheses.

† Significantly different from controls at $P<0·05$ (Mann–Whitney non-parametric rank test).

‡ Significantly different from controls at $P<0·001$ (Mann–Whitney non-parametric rank test).

PB was similar for both groups and without significant statistical difference (control=0·88 (range 0·2–3·7); MVV=0·86 (range 0·3–2·2) (results expressed as median of percentages of positively labelled cells in lymphocyte gate)).

Macrophage phenotyping in BALF

Most of the macrophages expressed LCA (CD45 analogue), but neither the control nor the MVV-infected animals showed expression of CD4, CD8, $\gamma\delta$ TCR, CD5 or IgG light chain molecules on the alveolar macrophage population.

MHC class II expression on macrophages and lymphocytes

The results of the expression of the MHC class II on alveolar macrophages are shown in Table 3 and Fig. 1. As these molecules are constitutively expressed on most of the ovine alveolar macrophages [22] no significant differences in the percentages of cells expressing MHC class II between the two groups of animals were revealed. However, MVV-infected animals showed a higher fluorescence intensity when compared with controls. By using the mode channel number, a significant increase in MHC class II molecule expression was observed on the alveolar macrophages of MVV-infected animals with the three reagents used. Similar studies on the lymphocyte populations of MVV-infected and control animals revealed no significant differences in the expression of these molecules (data not shown).

LFA-1 and LFA-3 expression on BALF lymphocytes and macrophages

The mode channel number was also used for the study of LFA-1 and LFA-3 as they were constitutively expressed on both lymphocytes and macrophages recovered from BALF. BALF lymphocytes from MVV-infected animals had significantly increased ($P<0·05$) LFA-1 (control=188·1 (range 47·7–311·9); MVV=255·8 (range 76·3–360·4)) and LFA-3 (control=42·8 (range 12·1–88·1); MVV=70·9 (range 51·3–126·5) (values expressed as median of the value of mode channel number)) (Fig. 2). Although alveolar macrophages from MVV-infected animals showed a higher expression of both molecules, no significant differences were found.

DISCUSSION

The characterization of the cellular phenotypes occurring in BALF during the course of MVV infection has not previously been described. The results shown in this study demonstrate alterations in the normal composition of CD4, CD8 and CD5 lymphocyte subsets and in the level of the expression of molecules that could play a key role in the pathogenesis of MVV in the lung and could provide a better understanding of the pathogenic mechanisms of lentiviruses.

The total BALF cell count in MVV-infected animals did not differ significantly from the controls. Considering that MVV lesions in lungs are characterized by an increased interstitial cellularity and a thickening of the alveolar septa [8] and also increased cellularity in BALF from MVV pathologically affected animals in slaughterhouses have been described [23],

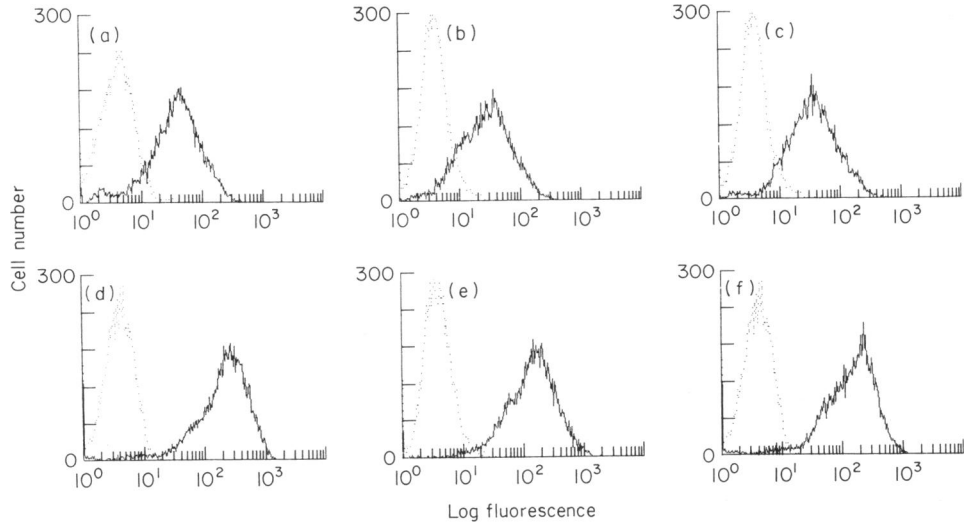

Fig. 1. Fluorescence histograms of MHC class II expression on bronchoalveolar lavage fluid (BALF) macrophages from control and maedi visna virus (MVV)-infected sheep. (a), (b), (c) Control animals. (d), (e), (f) MVV-infected sheep. (a), (d) Pan class II (VPM 36 and VPM 38). (b), (e) VPM 37 (DR). (c), (f) VPM 41 (DQ). (·····), Negative control. Fluorescence profiles are shifted to the right in MVV-infected animals for the three reagents used.

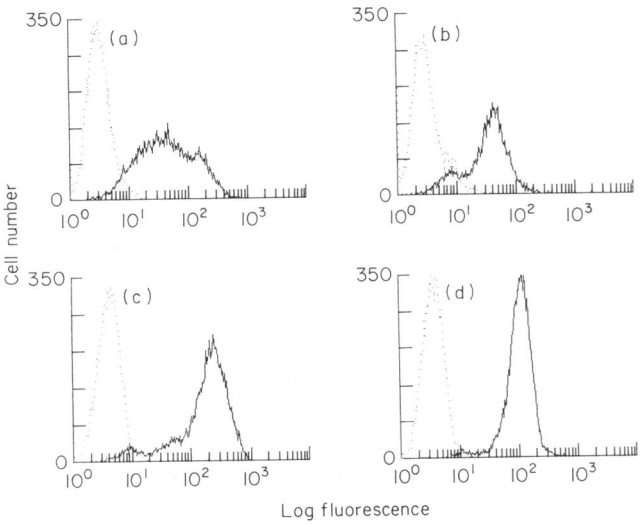

Fig. 2. Fluorescence histograms of LFA-1 and LFA-3 expression on bronchoalveolar lavage fluid (BALF) lymphocytes from control and maedi visna virus (MVV)-infected sheep. (a), (b) Control animals. (c), (d) MVV-infected sheep. (a), (c) LFA-1. (b), (d) LFA-3. (·····), Negative control. Fluorescence profiles are shifted to the right in MVV-infected animals in both cases.

these data could appear paradoxical. However, as we previously mentioned, our study is a cross section of an infected flock with animals in different stages of the disease (Collie *et al.*, manuscript accepted). This increase in the total cell count, even though not significant, seems to indicate a progressive tendency to an accumulation of cells in the lung interstitium.

A marked increase of BALF lymphocytes and neutrophils and a slight increase of macrophages in MVV-infected animals were the most noticeable results. Lymphocytes are the main mononuclear cell type found in MVV lung lesions [8] and an increased recovery of these cells can be expected. On the other hand, an increased secretion of Neutrophil Chemotactic Activity (NCA) by alveolar cells from MVV pathologically affected animals from slaughterhouses has been reported [23] and this could account for the increased recruitment of neutrophils into the affected lungs. However, the pathogenic role of these neutrophils, if any, remains to be determined. The increased percentage of neutrophils and lymphocytes seen in BALF of MVV-infected animals appeared to be a local phenomenon within the lung as none of these animals had elevated numbers of circulating neutrophils or lymphocytes. These findings are concordant with many other interstitial lung diseases in humans and animals [24].

The significant decrease ($P<0{\cdot}001$) in the $CD4^+/CD8^+$ ratio of lymphocytes from BALF of MVV-infected animals is due to both a significant increase of $CD8^+$ T lymphocytes and a significant decrease of $CD4^+$ T lymphocytes. Similar findings have been found in synovial fluid of sheep with MVV-induced arthritis [25] and in humans with AIDS-related lymphoid interstitial pneumonia [26]. However, the total depletion of $CD4^+$ T lymphocytes that characterizes AIDS and predisposes to opportunistic infections [27] is not found during the course of MVV infection, the $CD4^+$ T cell population being significantly decreased, but never absent. This finding would suggest that although lentiviruses both in humans and sheep induce inflammatory changes by similar mechanisms, they differ in some aspects of their pathogenesis. Further investigations are required to determine if the decrease in $CD4^+$ T lymphocytes reflects an infection in some cells of this lymphocytic subset. However, recent work demonstrated that sheep lentivirus is not lymphocytotropic and does not replicate productively in $CD4^+$ T lymphocytes obtained from blood and lymph [28]. Therefore, other explanations are needed for the $CD4^+$ T cell reduction. The decrease of $CD5^+$ cells seems to indicate lymphocyte activation as previous workers have reported an almost total loss of cell membrane $CD5^+$ expression during lymphocyte activation, as previous workers have reported an almost total infected animals. The biological role of these cells in the ruminant immune system remains unclear, although protection of epithelial surfaces has been proposed [29]. The fact that MVV does not involve epithelial damage in the lungs during its pathogenesis, could account for the lack of changes seen in $\gamma\delta$ T cells in BALF from MVV-infected animals.

Alterations in the distribution of lymphocyte phenotypes in BALF from MVV-infected animals were distinct from and not correlated with lymphocyte phenotype changes in PB. However, correlated changes between PB lymphocytes and synovial fluid lymphocytes in MVV-infected-cachexic animals have been observed [30]. It is known that blood lymphocytes do not necessarily reflect pathological changes in other organs/tissues [31] and previous studies have shown lack of correlation between alterations in lymphocyte subsets in blood and those in BALF [32].

Cell surface phenotypes of ovine bronchoalveolar macrophages from control and MVV-infected animals were similar, with expression of LCA and lack of CD4, CD8, CD5 and $\gamma\delta$ TCR. Similar results have been described in healthy sheep [22]. However, expression of CD4 and CD8 molecules on cultured ovine macrophages derived from blood monocytes from both MVV-infected and control animals has been observed (Wei-Cheng Lee, personal communication).

The two- to four-fold greater expression of MHC class II on alveolar macrophages of MVV-infected animals and the increased expression of LFA-1 and LFA-3 molecules on lymphocytes can account for the pathogenesis of the lymphoproliferative response and viral persistence. It has been demonstrated that class II antigens act as a component of the cellular receptor for MVV [33], and hence an increase of these molecules could promote the spread of the infection to uninfected macrophages, allowing persistence within the host. MVV-infected macrophages also induce the release of a specific interferon by T lymphocytes that is essential for the persistent expression of MHC class II antigens on the alveolar macrophages [34,35]. The continuous presence of these antigens could promote constant lymphocyte recruitment into the lungs, mediating local lesions and tissue damage. Furthermore, the increased expression of LFA-1 and LFA-3 in BALF lymphocytes of MVV-infected sheep would also contribute to the accelerated entry and accumulation of lymphocytes into the lung interstitium during the disease pathogenesis. LFA-1 and LFA-3 are cell adhesion molecules with a key role in leucocyte trafficking, activation and proliferation [36]. The increased expression of these molecules could have an important role as factors favouring the entrance and retention of new lymphocytes, their activation *in situ*, and their proliferation within the lungs, ending with the interstitial lymphoid proliferation seen in

the pulmonary parenchyma of MVV-infected animals. More studies are needed to understand the exact role of these and other adhesion molecules in the pathogenesis of the ovine lymphoid interstitial pneumonia.

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