Detection of bovine viral diarrhoea virus p80 protein in subpopulations of bovine leukocytes

P. Sopp,* L. B. Hooper, M. C. Clarke, C. J. Howard and J. Brownlie

AFRC Institute for Animal Health, Compton Laboratory, Compton, Newbury, Berkshire, RG16 0NN, U.K.

Flow cytometry and two-colour immunofluorescence were used to detect cytoplasmic bovine viral diarrhoea virus (BVDV) antigen in leukocytes from viraemic cattle. Monoclonal antibody to the p80 protein of BVDV, a non-structural viral antigen, was used to identify the subpopulations of leukocytes in which viral protein synthesis had occurred. Viral antigen was detected in 23% of peripheral blood mononuclear cells. Monocytes were found to have the highest frequency of infection (35%). A higher proportion of $CD2^+$ T cells (23%) were infected, compared with B cells (11%) or $WC1^+$ $\gamma\delta$ T cells (11%). No significant differences in percentages of different leukocyte subpopulations in blood were detected in persistently viraemic animals compared with controls.

Exposure of the bovine foetus to non-cytopathogenic bovine viral diarrhoea virus (BVDV) before immunocompetence can result in specific immunotolerance to the virus and the birth of calves that are persistently viraemic (Brownlie, 1991). Such viraemic calves can be either the offspring of seronegative cattle that were exposed to the virus before 110 days of gestation or the offspring of dams that were themselves persistently infected. Superinfection of these viraemic cattle by cytopathogenic strains of BVDV can lead to the development of mucosal disease (Brownlie *et al.*, 1984).

BVDV replicates in a wide range of cell types *in vitro* and has been identified *in vivo* in many tissues of persistently infected animals (Fernandez *et al.*, 1989; Bielefeldt Ohmann, 1988). There are several reports of the virus infecting leukocytes (Bielefeldt Ohmann *et al.*, 1987; Jensen *et al.*, 1989) which could lead to defective immune responses and play a role in the tolerance. Recently, flow cytometry has been used to investigate the extent of BVDV infection in leukocyte subpopulations and this technique has been proposed as a rapid assay for the detection of persistently infected cattle (Qvist *et al.*, 1990; Wolf & Rademacher, 1992). Two-colour immunolabelling systems for the simultaneous detection of BVDV antigen and molecules expressed on the surface of leukocytes were developed for light microscopy (Bielefeldt Ohmann, 1987) and showed that viral antigen was present in monocytes, T and B cells. Viral antigen was detected with polyclonal antibodies and its presence may have resulted from viral protein replication or from uptake of virions. By selecting a monoclonal antibody (MAb) to the non-structural viral polypeptide p80, a product of BVDV replication (Dubovi, 1990) and using this in a two-colour immunolabelling system for flow cytometry with MAbs to leukocyte surface antigens, we have been able accurately to identify and quantify cells in which the virus is replicating.

Four MAbs specific to BVDV were assessed; WB103 and WB112 recognize the p80 non-structural polypeptide; WB162 and WB214 recognize the gp53 structural polypeptide (Edwards *et al.*, 1991). The optimal concentrations of antibodies for staining BVDV antigen by flow cytometry was determined by titration on calf testis cells infected with a non-cytopathogenic strain (Pe515) of BVDV (Brownlie *et al.*, 1984). WB103 was selected for use as it gave the most intense staining. The MAbs to cell surface antigens are listed in Table 1. Optimal concentrations were determined by titrations on peripheral blood mononuclear cells (PBMCs) from normal healthy cattle; MAbs TRT1 (IgG1) and TRT6 (IgG2a) which recognize turkey rhinotracheitis virus were used as 1:1000 dilutions of ascitic fluids to monitor non-specific binding to the cell membrane and cytoplasm. All MAbs were of murine origin and were diluted in staining medium, RPMI-1640 (Gibco) supplemented with 10% (v/v) fetal calf serum (FCS), 25 mM-HEPES and 0·1% (w/v) sodium azide. The FCS was screened and shown to be free of BVDV and BVDV antibody. Biotinylation of MAbs was performed as described previously (Sopp *et al.*, 1991).

The cattle were Friesian/Aberdeen Angus crossbreeds. Eight viraemic animals were used. Five were over 1 year old of which three were naturally infected and two were the offspring of seronegative dams that had been

0001-2034 © 1994 SGM

Table 1. *MAbs to cell surface antigens*

MAb	Isotype	Specificity	Reference
CC42	IgG1	CD2	Howard & Morrison (1991).
IL-A43	IgG2a	CD2	Howard & Morrison (1991).
CC8	IgG2a	CD4	Howard & Morrison (1991).
CC63	IgG2a	CD8	Howard & Morrison (1991).
CC15	IgG2a	WC1*	Clevers *et al.* (1990).
CC21	IgG1	WC3†	Naessens *et al.* (1990).
IL-A65	IgG2a	WC3	Naessens *et al.* (1990).
IL-A58	IgG2a	Ig	Williams *et al.* (1990)
CC108	IgG1	MHC II	C. J. Howard (unpublished)
IL-A21	IgG2a	MHC II	Baldwin *et al.* (1988).
CC84	IgG1	Mo‡ and PMN§	Hall *et al.* (1993).
IL-A24	IgG2a	Mo and PMN	Ellis *et al.* (1988).

* WC1, 215K antigen present on $\gamma\delta$ T cells.
† WC3, 145K antigen present on B cells.
‡ Mo, monocytes.
§ PMN, Polymorphonuclear neutrophils.

Table 2. *Subpopulations of leukocytes in viraemic (BVDV) and control animals aged about 1 year*

		PBMCs stained (%)			
		BVDV		CONTROL	
MAb	Specificity	Mean ($n = 5$)	S.D.	Mean ($n = 5$)	S.D.
CC8	CD4	25	7·3	23	5·2
CC63	CD8	21	1·5	19	4·8
CC42	CD2	47	9·1	49	9·6
CC15	WC1*	19	6·1	18	7·5
CC21	WC3*	24	7·1	26	4·4
IL-A24	Monocytes	10	2·2	7	3·2
IL-A21	MHC II	34	6·4	34	6·0

* As defined in Table 1 footnote.

infected intranasally with Pe515 before 90 days of gestation. These five animals were sampled three times over a period of 5 months. Three other calves first examined when aged between 70 and 108 days were sampled again after a further 27 days. These three animals were the offspring of dams infected intranasally with Pe515. Five normal non-viraemic animals over 1 year old were used as controls. PBMCs were obtained by density gradient centrifugation using Histopaque 1·083 g/l (Sigma).

The procedure for permeabilization was a modification of the method of Labalette-Houache *et al.* (1991). Briefly, 2×10^7 PBMCs were washed three times in isotonic saline at 4 °C, pelleted by centrifugation at 300 ***g*** for 8 min, then resuspended in 6 ml of cold lysophosphatidylcholine (lysolecithin; Sigma) at 5 µg/ml in 0·02 M-sodium acetate, adjusted to pH 4·5 with 0·02 M-citric acid. The suspension was held on ice for 2 min. Permeabilization was stopped by the addition of 2 ml of cold paraformaldehyde (Sigma), 4% (w/v) in PBS. After a further 5 min incubation the cells were washed three times in wash buffer, PBS containing 1% (w/v) BSA or FCS and 0·1% (w/v) sodium azide. Efficiency of permeabilization was assessed by incubating treated PBMCs with propidium iodide (Sigma), 10 µg/ml in PBS. The proportion of fluorescing cells was calculated with a FACScan (Becton Dickinson). Routinely, about 95% of cells were permeabilized and > 90% were recovered.

Immunofluorescent staining and flow cytometric analysis were performed for simultaneous detection of cytoplasmic p80 antigen and leukocyte surface antigens. Permeabilized cells (10^6) in 100 µl staining medium were added to each well in a microtitre plate. After centrifugation at 300 ***g*** for 2 min, 25 µl of WB103 (IgG1) and 25 µl of MAb to a leukocyte surface antigen (IgG2a) were added to the wells and the plates were incubated at 20 °C for 10 min. Cells were washed twice in wash buffer, then 50 µl staining buffer containing 1:200 goat anti-mouse IgG1 fluorescein isothiocyanate (FITC) and 1:500 goat anti-mouse IgG2a phycoerythrin (PE) conjugates (Southern Biotechnology Associates) was added. After 10 min incubation at 20 °C the cells were washed twice in wash buffer and fluorescent staining was assayed using a FACScan. The percentage of each leukocyte subpopulation positive for BVDV p80 was calculated by quadrant analysis using FACScan Research Software (Becton Dickinson). An alternative staining protocol utilizing biotinylated MAbs was used initially, adapted from Sopp *et al.* (1991).

The relative proportions of subpopulations of leukocytes in PBMCs from five viraemic and five similarly aged normal animals were investigated by single-colour indirect immunofluorescent staining of non-permeabilized PBMCs, essentially as described above, but using goat anti-mouse Ig–FITC conjugate (Southern Biotechnology Associates). No significant differences (Table 2) were found ($P > 0·09$; Student's *t*-test) in the percentages of B cells, monocytes or the $CD4^+$, $CD8^+$ and $WC1^+$ T cells when the two groups of animals were compared with a range of MAbs (Table 1).

The preliminary study that used streptavidin–PE to detect biotinylated antibodies resulted in very high background staining of permeabilized cells. The streptavidin apparently bound directly to cytoplasmic biotin or non-specifically to other cytoplasmic components (Fig. 1 *i*). The quality of staining was not improved by various blocking methods. The procedure using isotype-specific fluorochrome conjugates described here did not give problems with non-specific staining. MAb to p80 gave a distinct intense staining of a subpopulation of PBMCs (Fig. 1 *h*). Four discrete populations of cells were seen in the bivariate displays of the staining for p80 and leukocyte surface antigens (Fig. 1 *b* to *g*, and *i*). These represented cells stained by MAbs to surface antigen

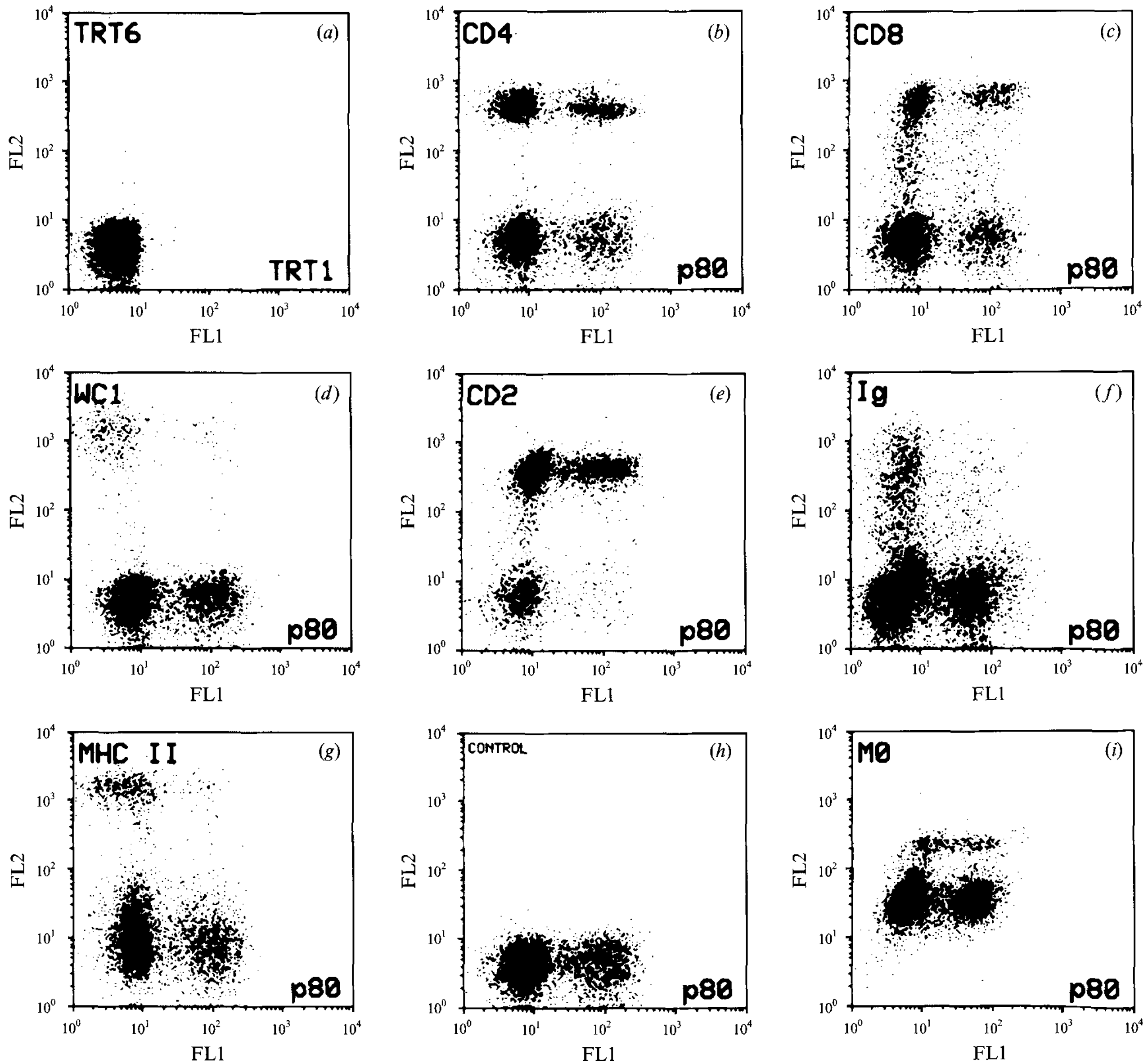

Fig. 1. Bivariate displays of two-colour immunofluorescent staining of PBMCs from an animal persistently infected with BVDV. Panels (*a*) to (*h*) are PBMCs stained with isotype-specific fluorochrome conjugates. Fluorescence 1 (FL1): (*a*) TRT1, isotype control; (*b* to *h*) BVDV p80 stained by MAb WB103. Fluorescence 2 (FL2): (*a*) TRT6 (IgG2a), isotype control; (*b*) CD4 stained by MAb CC8; (*c*) CD8 stained by MAb CC63; (*d*) WC1 ($\gamma\delta$ T cells) stained by MAb CC15; (*e*) CD2 stained by MAb IL-A43; (*f*) Ig (B cells) stained by MAb IL-A58; (*g*) MHC class II stained by MAb IL-A21; (*h*) reagent control. (*i*) WB103 stained with goat anti-mouse Ig–FITC (FL1) and monocytes identified by biotinylated MAb CC84, stained with streptavidin–PE (FL2).

only (top left), cells stained by MAb to p80 only (bottom right), cells unstained by both MAbs (bottom left) and doubly stained cells positive for surface antigen and p80 (top right). Staining for p80 was detected only in the permeabilized cells from the infected animals; cells that had not been permeabilized did not stain (data not shown). Non-specific binding by isotype controls was not observed (Fig. 1*a*).

The p80 antigen was detected in a subset of each of the leukocyte populations investigated (Table 3, Fig. 1). The percentage of p80$^+$ PBMCs varied considerably between animals (12 to 39%, Table 3). However, for individual animals the percentage of p80$^+$ leukocytes remained remarkably constant on each occasion on which blood was examined. Monocytes were the population with the highest proportion (mean of 35%) of p80$^+$ cells. A higher percentage of CD2$^+$ T cells (23%) were p80$^+$, compared with WC3$^+$ B cells (11%) or WC1$^+$ $\gamma\delta$ T cells (11%) (Table 3). There was considerable variation in the number of infected cells within the MHC II$^+$ subpopul-

Table 3. *Percentages of p80⁺ cells in subpopulations of PBMCs from five viraemic cattle*

		Animal									
		0825		1035		1134		1263		1279	
MAb	Specificity	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
NA*	PBM	12†	1·3	25	3·0	39	5·4	21	7·1	20	3·1
CC8	CD4	10	2·0	32	2·3	42	6·8	23	10·5	22	1·8
CC63	CD8	13	0·8	34	0·8	48	10·1	22	8·3	29	3·9
IL-A43	CD2	12	1·9	33	2·6	45	7·3	23	8·4	25	3·2
CC15	WC1‡	4	0·2	13	1·8	30	5·7	6	3·2	5	1·9
IL-A65	WC3‡	4	1·3	11	1·0	15	0·5	14	0·2	11	2·2
IL-A24	Monocytes	38	NA	41	NA	42	NA	27	NA	27	NA
IL-A21	MHC II	14	5·2	16	4·2	25	7·9	24	4·4	10	1·9

* NA, Not applicable.
† p80⁺ cells within indicated population (%); $n = 3$, except for monocytes ($n = 1$) and WC3 ($n = 2$).
‡ As defined in Table 1 footnote.

Table 4. *Percentages of p80⁺ cells in PBMCs of young viraemic calves*

Animal	2350		2397		2401	
Age (days)	108	135	71	98	70	97
PBMC p80⁺ (%)	20	17	< 1	< 1	44	37

ation. MHC class II is expressed by B cells, monocytes and a few CD2⁺ T cells in normal bovine PBMCs and therefore included the cells that contained the highest and lowest p80⁺ subsets. Calculations based on the subpopulations of PBMCs from viraemic cattle (Table 2) and the group mean of percentages of cells that were p80⁺ (Table 3) showed that approximately 31% of the p80⁺ cells were CD4⁺, 29% were CD8⁺, 10% were WC1⁺ ($\gamma\delta$ T cells), 13% were WC3⁺ (B cells) and 17% were IL-A24⁺ (monocytes). In the young calves p80 was detected in PBMCs from two of the three animals (Table 4). No significant differences were found ($P > 0{\cdot}18$; Student's *t*-test) in the percentages of leukocyte subpopulations infected in these two animals (data not shown) when compared with the older animals (Table 3).

The permeabilization method allowed intense staining of the BVDV p80 antigen. Staining was evident only when the cells from the viraemic animals were permeabilized, demonstrating that the p80 antigen was intracellular and since the antigen is non-structural, staining indicated cells in which viral protein synthesis had taken place. It might be expected that intermediate levels of fluorescence would be detected in newly infected cells where accumulation of viral product was submaximal. However, this was not seen and stained cells were clearly distinguished from unstained cells. This observation may result from the early expression of p80 in the replication cycle. Alternatively, replication of BVDV may occur when the cells are in the body tissues before they enter the circulation.

We found no evidence for age of the animals influencing the proportions of subpopulations infected. However, positive cells were not detected in one animal. When the samples were collected this calf had a viraemia of low titre and maternal antibody to BVDV was detected which may explain the failure to detect p80⁺ cells.

Data from the two-colour staining showed that viral protein synthesis can occur in all major cell subpopulations of PBMCs but the percentage of cells infected varied within the different subpopulations. More monocytes (mean of 35%) and CD2⁺ T cells (23%) were infected compared to WC1⁺ $\gamma\delta$ T cells (11%) and WC3⁺ B cells (11%) in each of the seven animals. Peripheral blood neutrophils were not investigated in this study; however, Wolf & Rademacher (1992) reported that a high percentage stained for viral antigen with MAb to p125/p80 indicating that viral protein synthesis also occurs in these cells.

Recently, Lopez *et al.* (1993) used flow cytometry and PCR techniques to investigate BVDV antigen in leukocyte subpopulations from naturally infected persistently viraemic animals. In contrast to our findings BVDV antigen was not detected in B cells. This may be due to the different sensitivity level of the technique used and the use of MAb against gp48 to detect BVDV antigen. In a comparison of MAbs directed against p80, gp53 and gp48, PBMCs from viraemic animals stained very weakly with MAb to gp48 (L. B. Hooper, unpublished). An alternative explanation is that the isolates studied varied in their tropism for different leukocytes.

Persistently viraemic cattle have been found to be slightly leukopenic compared to controls. In one study of cattle significantly fewer WC1⁺ PBMCs were found in viraemic animals compared with age-matched controls but there were no significant differences in the percentage of CD4⁺ or CD8⁺ T cells (Howard, 1990). Two comparisons have been made of sheep persistently

infected with border disease virus and normal animals. Burrells *et al.* (1989) found significantly increased numbers of B cells and although the percentage of T cells decreased, absolute numbers in the circulation were not affected in viraemic animals. Woldehiwet & Sharma (1990) reported increased numbers of $CD8^+$ and $CD5^+$, $CD4^-$, $CD8^-$ cells in peripheral blood.

The percentage of $WC1^+$ T cells in PBMCs varies considerably with age (Clevers *et al.*, 1990). About 25% of PBMCs in young animals are $WC1^+$; this is reduced to about 5% in adult cattle. Much smaller changes are evident in the percentage of $CD4^+$ and $CD8^+$ T cells. Infection may result in the selective changes in the ratios of particular subpopulations of PBMCs. Naessens & Williams (1992) noted that following infection with *Trypanosoma congolense* the percentage of $CD5^+$ B cells was markedly increased. Thus, to make meaningful relative comparisons of subpopulations of PBMCs it is necessary to control conditions exactly for age and husbandry. We found no significant differences in the leukocyte subpopulations of viraemic animals compared with age-matched controls. As some of the results reported are conflicting, we conclude that extensive investigations with carefully matched animals will be required to establish the true effect of persistent BVDV viraemia on PBMCs.

Fetal tolerance and persistence of viraemia are central to the pathogenesis of mucosal disease. The basis of this tolerance is still not certain but virus infection in many of the recirculating leukocytes, described in this report, provides a continuing source of viral antigen that could lead to central or peripheral tolerance. In persistent infections the mechanisms by which BVDV enters cells are unknown. If transmission of virus is dependent on cellular expression of a specific receptor they are clearly not lineage-restricted as subpopulations of all major leukocyte subpopulations are infected. Many monocytes and granulocytes in PBMCs will be recent emigrants from bone marrow, suggesting infection may at least in part be maintained from stem cells localized there. However, as many myeloid cells in PBMCs appear uninfected either all of the stem cells are not infected or viral protein synthesis may occur only in some cells although all cells might carry viral nucleic acid.

We are grateful to Mrs B. V. Jones for the production of MAbs TRT1 and TRT6, Dr S. Edwards for supply of MAbs WB103, WB112, WB162 and WB214 and to the International Laboratory for Research on Animal Diseases, Nairobi, Kenya for supply of antibodies IL-A43, IL-A65, IL-A58, IL-A21 and IL-A24.

References

BALDWIN, C. L., MORRISON, W. I. & NAESSENS, J. (1988). Differentiation antigens and functional characteristics of bovine leucocytes. In *Differentiation Antigens in Lymphohemopoietic Tissues*, pp. 445–498. Edited by M. Miyasaka & Z. Trnka. New York: Marcel Dekker.

BIELEFELDT OHMANN, H. (1987). Double-immunolabeling systems for phenotyping of immune cells harbouring bovine viral diarrhoea virus. *Journal of Histochemistry and Cytochemistry* **35**, 627–633.

BIELEFELDT OHMANN, H. (1988). BVD virus antigens in tissues of persistently viraemic clinically normal cattle: implications for the pathogenesis of clinically fatal disease. *Acta veterinaria scandinavica* **29**, 77–84.

BIELEFELDT OHMANN, H., RØNSHOLT, L. & BLOCH, B. (1987). Demonstration of bovine viral diarrhoea virus in peripheral blood mononuclear cells of persistently infected, clinically normal cattle. *Journal of General Virology* **68**, 1971–1982.

BROWNLIE, J. (1991). The pathways for bovine virus diarrhoea virus biotypes in the pathogenesis of disease. *Archives of Virology* supplementum 3, 79–96.

BROWNLIE, J., CLARKE, M. C. & HOWARD, C. J. (1984). Experimental production of fatal mucosal disease in cattle. *Veterinary Record* **114**, 535–536.

BURRELLS, C., NETTLETON, P. F., REID, H. W., MILLER, H. R. P., HOPKINS, J., MCCONNELL, I., GORRELL, M. D. & BRANDON, M. R. (1989). Lymphocyte subpopulations in the blood of sheep persistently infected with border disease virus. *Clinical and Experimental Immunology* **76**, 446–551.

CLEVERS, H., MACHUGH, N. D., BENSAID, A., DUNLAP, S., BALDWIN, C. L., KAUSHAL, A., IAMS, K., HOWARD, C. J. & MORRISON, W. I. (1990). Identification of a bovine surface antigen uniquely expressed on $CD4^-$ $CD8^-$ T cell receptor $\gamma\delta$ T lymphocytes. *European Journal of Immunology* **20**, 809–815.

DUBOVI, E. J. (1990). Molecular biology of bovine virus diarrhoea virus. *Revue Scientifique et Technique, Office International des Epizooties* **9**, 105–114.

EDWARDS, S., MOENNIG, V. & WENSVOORT, G. (1991). The development of an international reference panel of monoclonal antibodies for the differentiation of hog cholera virus from other pestiviruses. *Veterinary Microbiology* **29**, 101–108.

ELLIS, J. A., DAVIS, W. C., MACHUGH, N. D., EMERY, D. L., KAUSHAL, A. & MORRISON, W. I. (1988). Differentiation antigens on bovine mononuclear phagocytes identified by monoclonal antibodies. *Veterinary Immunology and Immunopathology* **19**, 325–340.

FERNANDEZ, A., HEWICKER, M., TRAUTWEIN, G., POHLENZ, J. & LIESS, B. (1989). Viral antigen distribution in the central nervous system of cattle persistently infected with bovine viral diarrhoea virus. *Veterinary Pathology* **26**, 26–32.

HALL, G. A., SOPP, P. & HOWARD, C. J. (1993). An investigation of temporary workshop clusters reacting with cells of the mononuclear phagocytic system. *Veterinary Immunology and Immunopathology* **39**, 225–236.

HOWARD, C. J. (1990). Immunological responses to bovine virus diarrhoea virus infections. *Revue Scientifique et Technique, Office International des Epizooties* **9**, 95–103.

HOWARD, C. J. & MORRISON, W. I. (editors) (1991). Leucocyte antigens in cattle, sheep and goats. *Veterinary Immunology and Immunopathology* **27**, 1–276.

JENSEN, J., AIKEN, J. & SCHULTZ, R. D. (1989). Detection of bovine viral diarrhoea virus genome in leucocytes from persistently infected cattle by RNA–cDNA hybridization. *Canadian Journal of Veterinary Research* **54**, 256–259.

LABALETTE-HOUACHE, M., TORPIER, G., CAPRON, A. & DESSAINT, J. P. (1991). Improved permeabilisation procedure for flow cytometric detection of internal antigens: analysis of interleukin-2 production. *Journal of Immunological Methods* **138**, 143–153.

LOPEZ, O. J., OSORIO, F. A., KELLING, C. L. & DONIS, R. O. (1993). Presence of bovine viral diarrhoea virus in lymphoid cell populations of persistently infected cattle. *Journal of General Virology* **74**, 925–929.

NAESSENS, J. & WILLIAMS, D. J. L. (1992). Characterisation and measurement of $CD5^+$ B cells in normal and *Trypanosoma congolense*-infected cattle. *European Journal of Immunology* **22**, 1713–1718.

NAESSENS, J., NEWSON, J., MACHUGH, N. D., HOWARD, C. J., PARSONS, K. & JONES, B. (1990). Characterisation of a bovine leucocyte differentiation antigen of 145,000 M_r restricted to B lymphocytes. *Immunology* **69**, 525–530.

QVIST, P., AASTED, B., BLOCH, B., MEYLING, A., RONSHOLT, L. & HOUE, H. (1990). Flow cytometric detection of bovine viral diarrhoea virus in peripheral blood leucocytes of persistently infected cattle. *Canadian Journal of Veterinary Research* **54**, 469–472.

SOPP, P., HOWARD, C. J. & PARSONS, K. R. (1991). Investigating monoclonal antibodies to bovine "null" cell antigens using two-colour immunofluorescence. *Veterinary Immunology and Immunopathology* **27**, 163–168.

WILLIAMS, D. J. L., NEWSON, J. & NAESSENS, J. (1990). Quantitation of bovine immunoglobulin isotypes and allotypes using monoclonal antibodies. *Veterinary Immunology and Immunopathology* **24**, 267–283.

WOLDEHIWET, Z. & SHARMA, R. (1990). Alterations in lymphocyte subpopulations in peripheral blood of sheep persistently infected with border disease virus. *Veterinary Microbiology* **22**, 153–160.

WOLF, G. & RADEMACHER, G. (1992). Rapid detection of bovine viral diarrhea virus in viraemic cattle. *Proceedings of XVII World Buiatrics Congress 1992, St Paul, Minnesota, U.S.A.*, **1**, 178–183.

(*Received 20 August 1993; Accepted 26 November 1993*)