Presence of bovine viral diarrhoea virus in lymphoid cell populations of persistently infected cattle

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Cattle infected in utero with bovine viral diarrhoea virus (BVDV) often develop a lifelong persistent infection (PI). During this PI, BVDV infects many cell types including peripheral blood mononuclear cells (PBMNC). To define the lymphoid cell populations in which BVDV persists PBMNC subpopulations were separated using monoclonal antibodies to cell surface markers. Separated cells were analysed by a sensitive PCR assay for BVDV, in conjunction with flow cytometry to identify antigen-containing cells and with viral infectivity assays. The results indicate that BVDV establishes a productive PI in monocytes and T cells bearing the marker BoCD4, BoCD8 or gamma–delta T cell receptor. BVDV was not detected in B cells as a productive nor a latent infection.

Bovine viral diarrhoea virus (BVDV) is one of the most important pathogens of cattle. Its genome is a single-stranded (positive polarity) non-polyadenylated RNA molecule of about 12.5 kb in length. BVDV and the other members of the Pestivirus group, border disease virus and hog cholera virus, have been classified as members of the family Flaviviridae (Collett et al., 1989; Horzinek, 1991).

Field BVDV isolates can be divided into two biotypes according to their cytopathic effect in cell cultures (Lee & Gillespie, 1957; Gillespie et al., 1960). Infection with the non-cytopathic biotype during early gestation leads to the birth of persistently infected (PI) calves (McClurkin et al., 1984). PI animals are immunologically tolerant to the non-cytopathic BVDV strain which infects them (McClurkin et al., 1984), but if superinfection with a related cytopathic biotype takes place, or if a cytopathic biotype arises from persisting BVDV, the animal will usually die of mucosal disease (Bolin et al., 1985a; Brownlie et al., 1984).

It has been demonstrated that calves infected with BVDV exhibit an alteration of some immune functions (Reggiardo & Kaeberle, 1981; Roth & Kaeberle, 1983; Brownlie et al., 1984; Roth et al., 1986; Brown et al., 1991) and this correlates with in vitro parameters (Atluru et al., 1979, 1990). Moreover, a link has been found between BVDV infection and enhancement of concurrent infections (Edwards, 1986).

BVDV has affinity for cells of the immune system either in acutely infected or PI animals (Bielefeldt Ohmann et al., 1987; Bolin et al., 1987; Bolin & Ridpath, 1990). Acute infection of calves produces a decrease in the number of leukocytes (Bolin et al., 1985a; Ellis et al., 1988), and BVDV antigen has been detected in PI cattle in T, B and null lymphocytes, as well as in monocytes (Bielefeldt Ohmann et al., 1987), platelets (Corapi et al., 1989) and many other cell types (Allan et al., 1989; Bielefeldt Ohmann, 1983). Presence of virus was also observed by electron microscopy of lymphoid cells (Bielefeldt Ohmann et al., 1988).

The mechanism that leads to tolerance in PI with BVDV and the kind of interaction that takes place between the virus and the cells of the immune system are unknown (Moennig, 1990). Increased knowledge of the infected cell populations, and the functional alterations caused by this infection, will help the understanding of the degree of immunosuppression caused by the viral infection in the acute, persistent and mucosal disease phases of the disease. We report a study of the cellular tropism of non-cytopathic BVDV in naturally PI cattle. We analysed five PI animals from three different herds for 8 months. The animals consisted of one 4-5 year old cow, a 1-5 year old calf, and two heifers and one steer of approximately 3 years of age. We focused on the detection of BVDV in purified lymphoid populations using techniques including detection of viral antigens by flow cytometry, isolation of infectious virus and detection of a conserved BVDV genomic sequence (Collett et al., 1988) by reverse transcription of the viral RNA followed by PCR (RT–PCR) (Lopez et al., 1991). As controls for

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these experiments we used peripheral blood mononuclear cells from two 9 month old calves from a beef herd raised at the Agricultural Research and Development Center of the University of Nebraska that is maintained free of BVDV, bovine herpesvirus 1 and bovine leukaemia virus infections. The control animals were maintained in isolation and monitored (by serology and viral isolation) for possible BVDV infection, throughout the whole experiment.

For positive selection and characterization of lymphoid populations we used a panel of monoclonal antibodies (MAbs) directed against the following specific bovine cell (Bo) surface markers: ILRAD 11 against BoCD4, ILRAD 15 against BoCD11b (Mac 1), ILRAD 21 against monomorphic determinant class II (Mo), ILRAD 29 against gamma–delta T cell receptor, ILRAD 30 against IgM (mu chain), ILRAD 42 against BoCD2 and ILRAD 51 againsty BoCD8. All of these MAbs belong to a collection specific for bovine leukocyte surface markers developed by the International Laboratory for Research in Animal Disease (ILRAD), Nairobi, Kenya.

Blood was collected, after venipuncture from the jugular vein, in tubes with 37.50 mg/ml EDTA. The tubes were centrifuged at 800 g for 20 min at 18 °C, and the buffy coat was extracted and diluted in Ca²⁺/Mg²⁺-free PBS. To separate mononuclear cells from other cell types, the buffy coat suspension was loaded onto Lymphopaque (density 1.086 g/ml; Nygaard and Co.), and centrifuged at 800 g for 20 min at 18 °C. After three washes with 1 % albumin (fraction V; Sigma) and 0.04 % sodium azide in PBS, 25 μg of purified MAb against the appropriate cell surface marker was added to 10⁶ mononuclear cells and incubated for 45 min at 4 °C. A goat anti-mouse IgG FITC conjugate (Boehringer-Mannheim) was used as second antibody. After staining, cells were fixed in 3 % formaldehyde in PBS for 1 h and washed four times in PBS. Cells were examined within 1 week for fluorescent emission in a Coulter 741 flow cytometer.

In order to study the extent of BVDV infection in peripheral lymphoid populations, different cell populations were purified by panning (Lewis & Kamin, 1980), as follows. Briefly, 5 x 10⁷ total peripheral blood mononuclear cells in 5 ml MEM plus 5 % fetal calf serum were loaded into Petri dishes coated with the MAbs directed against the appropriate cell surface markers as described previously, and incubated for 1 h at 4 °C. After rinsing with PBS to remove the floating cells, the attached cells were released by gentle scraping and washed twice in MEM plus 5 % fetal calf serum. The efficiency of purification was over 97 % in every case, as assessed by cell surface fluorescence. The presence of BVDV in the unfractonated or separated mononuclear cell populations was assayed by BVDV antigen detection, isolation of infectious BVDV and detection of viral RNA.

In order to determine the proportion of productively BVDV-infected lymphoid cell populations we carried out semi-quantitative infectivity studies on positively selected single-cell populations. We used bovine testicle (BT) cells to detect the release of infectious BVDV from separated lymphoid populations from three PI animals. The BT cell line (established by R. O. Donis & E. J. Dubovi, New York State Diagnostic Laboratory, Cornell University, Ithaca, N.Y., U.S.A.) is free of BVDV and mycoplasma infections.

Decreasing 10-fold dilutions (range 10² to 10¹) of separated cells from PI animals were loaded onto four 32 mm well plates with BT cells. It was not necessary to assay more than 10⁶ cells because they were over 95 % pure. After two consecutive passages, BT cells were trypsinized and loaded onto slides coated with polylysine (1 mg/ml) for 1 h at 4 °C and fixed for 10 min in acetone, at room temperature. An indirect fluorescence assay using MAb 15C5 (see below) or bovine hyperimmune serum, anti-BVDV (NADL), as first antibodies was performed. In three PI animals from three different herds, between 100 and 1000 purified monocytes or T cells were enough for detection of BVDV. However, up to 10⁵ B cells were consistently negative by this procedure (Table 1).

<table>
<thead>
<tr>
<th>Cell marker recognized by MAb</th>
<th>Antigen (%)</th>
<th>Minimum number of cells required for BVDV detection</th>
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<tbody>
<tr>
<td>CD2* (ILRAD 42)</td>
<td>PI 6.16±2.25</td>
<td>10⁴</td>
</tr>
<tr>
<td>CD3* (ILRAD 30)</td>
<td>PI 0.72±0.40</td>
<td>10³</td>
</tr>
<tr>
<td>CD4* (ILRAD 11)</td>
<td>PI 5.67±2.94</td>
<td>10⁴</td>
</tr>
<tr>
<td>CD8* (ILRAD 51)</td>
<td>PI 6.55±2.56</td>
<td>10³</td>
</tr>
<tr>
<td>Mo (ILRAD 21)</td>
<td>PI 10.19±4.39</td>
<td>10³</td>
</tr>
<tr>
<td>Gamma-delta (ILRAD 29)</td>
<td>PI 5.46±0.98</td>
<td>10³</td>
</tr>
<tr>
<td>Total (unrelated MAb)</td>
<td>PI 8.71±2.93</td>
<td>10³</td>
</tr>
</tbody>
</table>

* Detection of BVDV antigen by flow cytometry. Mean values (%) of fluorescent cells are given for PI and control (C) groups.
† Infectious virus.
‡ Detection by PCR.
§ Negative in 10⁶ cells.
∥ MAb against murine light (kappa) immunoglobulin chain.
A panel of MAbs developed against the Singer strain and specific for gp54 and gp48 (Donis et al., 1988; Collett et al., 1989) was assayed for immunofluorescence on BT cells infected with buffy coat and sera from the PI animals. Out of 11 MAbs, only one (15C5) reacted with the isolates from the three different herds. This MAb reacted with gp48 of BVDV.

Total or purified single lymphoid cell populations were fixed with 3% formaldehyde in PBS for 1 h at room temperature and were then washed and resuspended in 100 mM-ammonium chloride for 10 min at room temperature. They were permeabilized by incubation for 30 min in 0.05% NP40 in PBS, washed four times in PBS and incubated with MAb 15C5 for 45 min at 4°C. After two washes in PBS, the samples were incubated for 30 min with FITC-conjugated anti-mouse IgG. After three washes, the stained cells were assessed by flow cytometric analysis within a week.

Viral antigens were detected in all peripheral mononuclear populations examined except in B cells (Table 1). The percentage of T cells containing BVDV antigen was similar (no significant differences, \( P < 0.05 \), were found) in total T cells and/or T4, T8, gamma-delta T cell populations. However, a higher proportion of monocytes (\( P < 0.05 \)) were positive for viral antigen than the other cell populations.

Our detection of BVDV in ILRAD 29+ cells (Table 1) confirms the results of another laboratory (Bielefeldt Ohmann et al., 1988). This population, originally described as a null cell type, because of the lack of typical B or T markers, is currently thought to carry the gamma-delta T cell receptor. Cells carrying this receptor constitute an important T cell subpopulation in the bovine species (Hein & MacKay, 1991).

To detect BVDV RNA in lymphoid cells, we performed specific reverse transcription coupled with DNA amplification of RNA obtained from different single cell populations. Known aliquots of 10^1 to 10^9 were digested for 10 min at room temperature with proteinase K (200 \( \mu \)g/ml) and 20 min at 56°C after the addition of SDS (0.2% w/v). This solution was extracted with phenol-chloroform and the RNA was precipitated with cold 100% ethanol and 3 N-sodium acetate pH 5.2 on solid CO₂ for 1 h. For the precipitation of viral RNA, 100 ng of DNA from uninfected BT cells was used as the carrier. The pellet was dried in a desiccator and resuspended in 10 \( \mu \)l of water. For PCR a pair of primers was used that amplified a segment of 207 bp at the 3' end of the BVDV genome (Lopez et al., 1991). Both primers (20 \( \mu \)mol) were added to the resuspended pellet, heated for 5 min at 70°C for RNA denaturation, and incubated at 42°C for 1 h after addition of 2 \( \mu \)l of Schimke solution (comprising 50 mM-DTT, 50 mM-MgCl₂, 350 mM-KCl, 400 mM-Tris–HCl pH 8), 2 \( \mu \)l of 2.5 mM-dNTPs (Pharmacia), 0.5 \( \mu \)l of RNasin (Promega; 28 units/\( \mu \)l) and 1 \( \mu \)l of avian myeloblastosis virus reverse transcriptase (Life Sciences; 19.6 units/ml). For the PCR assay 5 \( \mu \)l of this solution was then used.

PCR was conducted as described previously (Lopez et al., 1991). Briefly, 10 \( \mu \)l of 10× amplification buffer, 16 \( \mu \)l of an equimolar mixture of dNTPs (5 mM), 3 \( \mu \)l of each primer (40 \( \mu \)mol) and 5 \( \mu \)l of reverse transcription solution were added, in a total volume of 100 \( \mu \)l. The mixture was heated at 95°C for 5 min, after which 3 units of Taq polymerase (Promega) in a 5 \( \mu \)l volume of 1× buffer was added. To prevent evaporation of reagents, 100 \( \mu \)l of Nujon mineral oil (Perkin Elmer) was added. The amplification reaction consisted of 40 equal cycles in an automated thermal cycler (Perkin-Elmer Cetus). Each cycle was composed of 1 min at 95°C for denaturation, 1 min at 55°C for primer annealing and 2 min at 72°C for primer extension. Several negative controls were added for detection of possible DNA carry over. A 30 bp probe was used for detection of specific amplified products after blotting of the electrophoresed gel. The oligonucleotide was end-labelled with ³²P and polynucleotide kinase. Viral RNA was then assayed in different purified single cell populations of the PI animals. In purified populations of T cells and monocytes, 1000 peripheral mononuclear cells were sufficient to detect the BVDV RNA, but not in the B cell fraction, where 10⁵ cells were consistently negative (Table 1).

To maximize the possibility of detection of minimal amounts of BVDV, we repeated the RT–PCR after two consecutive passages of B cells co-cultured with BT cells. BVDV RNA was then found in RNA extracted from the BT cells co-cultured with the B cell fraction (data not shown). To determine whether this result was due to infectious particles released upon productive cell infection or instead to extracellular virus physically adsorbed to B cells, we performed experiments in which peripheral mononuclear cells from the PI animals were treated with trypsin after purification and immediately before initiation of the co-cultures, as described by Bolin & Ridpath (1990). Total and purified lymphoid cells were resuspended in MEM plus 1% trypsin and were incubated for 30 min at 37°C (Bolin & Ridpath, 1990). MEM with 10% FCS was added after incubation and the suspension was centrifuged for 10 min at 800 g. The viability of the cell suspension as determined by trypan blue staining was greater than 80%.

Viral RNA was detected in cells co-cultured with the non-trypsin-treated B cells and in the non-B (T with monocytes) cells (B-depleted). At the same time, viral RNA was detected in the B-depleted fraction pretreated with trypsin, but not in the trypsin-treated purified B fraction.

In summary, we have detected BVDV antigen,
infectious particles and BVDV RNA in T cell populations and monocytes of peripheral blood from BVDV PI cattle, but only a small amount of BVDV can be detected in association with the B cells of these animals. This BVDV infectivity, which can be removed by enzymatic digestion of the B cells, probably represents BVDV adsorbed externally to the B cells instead of virus produced endogenously by replication.

Our inability to detect BVDV infection in B cells of PI animals contrasts with reports from other laboratories (Bielefeldt Ohmann et al., 1988; Bolin et al., 1987). Some authors have found infectious particles in purified B cells (Bolin et al., 1987) although others have not (Bielefeldt Ohmann et al., 1987). The contrasting results could be based on the use, by different laboratories, of different cell separation/purification techniques. Bolin et al. (1987) purified B cells by positive selection using a polyclonal serum against bovine IgG and then tested the purified single-cell population for infectivity on indicator cells. In contrast, Bielefeldt Ohmann et al. (1987) attempted to isolate virus from B cells negatively selected using a panel of MAbs, but subsequent co-cultivation yielded no virus. Here, we purified B cells by positive selection using an anti-mu chain MAb.

Our failure to detect viral antigen in B cells also contrasts with a report (Bielefeldt Ohmann et al., 1987) where total peripheral blood cells were centrifuged onto slides and a double staining technique was used to detect cell markers with specific MAbs and viral antigens with a BVDV-specific polyclonal serum. It could be argued that our failure to detect antigen in B cells could be caused by a lower sensitivity of our anti-BVDV gp48 MAb, which recognizes a single structural protein (Donis et al., 1988), in contrast to the polyclonal serum used by Bielefeldt Ohmann et al. However, our results are supported by our simultaneous failure to detect viral RNA in the same population using the highly sensitive PCR.

The difference between studies could also be related to tropism differences between our isolates and those of others. Our BVDV isolates are different from the Singer strain when tested against a panel of MAbs (data not shown). Finally, B cells may be differentially resistant to BVDV, depending on the physiological status of the PI animal, and/or the developmental stage at which the fetal infection (leading to PI) took place.

Although further studies on BVDV genome transcription and expression in lymphoid cells would be pertinent in order to ascertain the exact type of infection established in these cells, our simultaneous detection of viral antigen, infectious particles and viral genome in the different populations suggests that in the PI cattle of our study, the BVDV–lymphoid cell interaction results in productive infection.

Whether B cells are always resistant to BVDV in vivo or their permissiveness for BVDV is related to the physiological status of the animal (or the viral strain causing PI) deserves further investigation. The inability of BVDV to infect B cells in naturally occurring PI cattle seems to indicate that there are restrictions to the generalized tropism of the virus. This merits study of the mechanism of BVDV penetration into specific lymphoid cells and investigation of B cell resistance. We do not know, however, when the block takes place although the failure to detect BVDV RNA in B cells suggests that B cells block BVDV infection at the penetration stage or an early stage of the infectious cycle, prior to viral protein synthesis.

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