Germinal centre localization of bovine viral diarrhoea virus in persistently infected animals

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Immunohistochemical analysis of peripheral lymph nodes from gnotobiotic calves persistently infected with bovine viral diarrhoea virus (BVDV) revealed extensive deposition of E\textsuperscript{ns} and localization of the viral genome in the light zone of germinal centres. Viral antigen co-localized with immunoglobulin in the germinal centres and was shown to be extracellular. Despite the presence of viral antigen in germinal centres, circulating anti-BVDV antibody was not detected. These findings provide evidence that calves persistently infected with BVDV, in the absence of adventitious infection, can generate a B cell response to the persisting virus. The nature of the tolerance in calves persistently infected with BVDV is discussed in light of these findings.

Bovine viral diarrhoea virus (BVDV) is a pestivirus belonging to the family \textit{Flaviviridae}. Pestiviruses are positive-stranded RNA viruses with a genome comprising a single open reading frame of approximately 12.5 kb. Two biotypes of BVDV, cytopathic (cp) and non-cytopathic (ncp), can be differentiated by their effect in cell culture (Bolin, 1995; Zhang \textit{et al.}, 1996). Infection of bovine foetuses with ncp BVDV during the first 120 days of pregnancy can result in the birth of persistently infected (PI) offspring that are apparently immuno-tolerant. These PI animals are viraemic and either have negative or low BVDV antibody titres (Duffell \& Harkness, 1985; Collins \textit{et al.}, 1999). It is unclear whether or not the reported low levels of antibody in some PI animals represent a response to the persistent virus or to an adventitious challenge with antigenically heterologous BVDV. Superinfection of PI animals with antigenically homologous cp BVDV results in fatal mucosal disease (Brownlie \textit{et al.}, 1984), which is characterized by destruction of gut-associated lymphoid tissues (Bolin, 1995; Fritzemeier \textit{et al.}, 1997).

Previous studies of animals infected with pestiviruses have highlighted the presence of the viral genome and/or antigen in the germinal centres of lymphoid tissue (Bielefeldt Ohmann, 1983, 1988; Collins \textit{et al.}, 1999; Desport \textit{et al.}, 1994; Susa \textit{et al.}, 1992). The classical swine fever virus (CSFV) genome has been localized to germinal centres after acute infection and, on the basis of this observation, it has been suggested that germinal centres are the site of CSFV replication (Susa \textit{et al.}, 1992). Sections of lymph nodes from cattle clinically affected by mucosal disease, probed with anti-BVDV polyclonal sera, showed heavy staining of germinal centres, particularly associated with the long cytoplasmic processes of large cells considered to be follicular dendritic cells (FDC) (Bielefeldt Ohmann, 1983). More recently, the BVDV genome and the glycoprotein E\textsuperscript{ns} have been localized to germinal centres in PI animals (Desport \textit{et al.}, 1994; Collins \textit{et al.}, 1999). More detailed aspects of the latter study (Collins \textit{et al.}, 1999) focussed on PI animals with detectable circulating anti-BVDV antibodies. In the present study, we set out to define the precise location of BVDV antigen and genome in the germinal centres of experimentally produced PI calves that did not have detectable circulating antibody to BVDV.

BVDV antibody-positive cows were presented for in utero infection at approximately 60 days of pregnancy (range 58–70 days): \(5 \times 10^{6} \text{ TCID}_{50}\) of ncp BVDV (Pe515ncp; Brownlie \textit{et al.}, 1984) was injected directly into the amniotic fluid. The cows were maintained in isolation from other cattle for the remainder of pregnancy. Calves were derived by hysterotomy at approximately 10 days before term into disease-secure accommodation, which ensured that there was no risk of extraneous infection. Each calf was confirmed to be persistently viraemic with BVDV and seronegative to BVDV (Brownlie \textit{et al.}, 1984). Prescapular lymph nodes were surgically removed at either 1 day of age (n = 2) or approximately 2 months of age (n = 4). Lymph nodes were also sampled from age-matched animals that had been shown to be negative for BVDV and antibody. Samples of each lymph node were either preserved in 4% paraformaldehyde for histological sectioning or mounted in OCT compound (Miles), frozen and stored at \(-70^\circ\text{C}\) for cryosectioning. \textit{In situ} hybridization of sections prepared from wax-embedded tissues was performed as described previously (Lennard \textit{et al.}, 1995), using an \(35\text{S}-\text{UTP}\)-labelled riboprobe transcribed from a 1.1 kb fragment of...
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E2 cDNA from Pe515ncp, cloned in pTarget (Promega). The distribution of BVDV antigens was examined by indirect immunofluorescence (IIF) (Fray et al., 1998) using three anti-BVDV MAbs, WB103, WB162 and WB210, which are specific for the non-structural protein NS3 and the envelope glycoproteins E2 and E\textsuperscript{rns}, respectively (Paton et al., 1997). Cryostat sections of prescapular lymph nodes from BVDV-positive and BVDV-free calves were stained with the MAbs and a control MAb of the same isotype (IgG1) raised against turkey rhinotracheitis virus-1, followed by FITC-labelled goat antimouse IgG. The control antibody did not give any staining in tissues from infected or uninfected animals and no staining was observed with the BVDV-specific antibodies in tissues from uninfected animals.

Histological comparison of lymph nodes from PI and uninfected calves did not reveal any gross differences in the structure of the follicles and germinal centres present. In sections of lymph node from the PI animals, individual cells staining for E2 and NS3 (Fig. 1 c, e, g) were observed scattered throughout the lymph node, including the B cell follicles, paracortex and medullary regions. There was also a similar distribution of cells that stained weakly for E\textsuperscript{rns} in addition to more intense and diffuse staining for E\textsuperscript{rns} within the germinal centres of the B cell follicles (Fig. 1 c). This latter staining was often focussed in the anterior pole of the germinal centre, proximal to the lymph node capsule. In situ hybridization showed that distribution of the BVDV genome was similar to that of E\textsuperscript{rns} with intense staining in the germinal centres of the B cell follicles (Fig. 1 a, b and c) as well as more widespread but weaker staining of individual cells. Compared to animals housed in a conventional environment, the lymph nodes from animals in this study contained relatively few germinal centres. The lymph nodes from both the PI and uninfected neonatal calves had very few primary and secondary B cell follicles. However, a large proportion of the secondary follicles from the PI animals exhibited diffuse staining for BVDV RNA and E\textsuperscript{rns} antigen in the light zone of their germinal centres. The lymph nodes from the two older calves examined in this study had a greater number of secondary B cell follicles than the neonatal calves, but only a minority of the germinal centres were positive for BVDV RNA and E\textsuperscript{rns} antigen. A positive-sense version of the E2 riboprobe did not hybridize to sections from viraemic animals. Sections from uninfected animals did not stain with either positive- or negative-sense riboprobes.

The distribution of staining for E\textsuperscript{rns} within germinal centres indicated that it was confined to the light zones, and the pattern of staining was similar to that observed for complexed immunoglobulin on FDC. Cryostat sections of lymph node were therefore examined by two-colour IIF to determine whether or not E\textsuperscript{rns} and bovine immunoglobulin co-localized. Sections were stained with the anti-bovine immunoglobulin light chain antibody, ILA 58 (isotype IgG2a; Williams et al., 1990) and the anti-E\textsuperscript{rns} MAb WB210 (isotype IgG1; Paton et al., 1997). The primary antibodies were localized with goat

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**Fig. 1.** (a)–(d) Examples of prescapular lymph node sections from PI animals stained for viral RNA and BVDV protein E\textsuperscript{rns}. (a) In situ hybridization showing localization of the BVDV genome in a lymph node B cell follicle. (b) In situ hybridization showing the distribution and detailed staining pattern of the BVDV genome in the germinal centre light zone of a B cell follicle. (c) Intense and diffuse staining for E\textsuperscript{rns} within the germinal centres of the B cell follicles. This staining was often focussed in the anterior pole of the germinal centre, proximal to the lymph node capsule. (d) Dual staining for E\textsuperscript{rns} with MAb WB210 and FITC-labelled (green) secondary antibody and anti-α-tubulin MAb with CYS-labelled (blue) secondary antibody. White colour indicates co-localization; the majority of the staining for E\textsuperscript{rns} did not co-localize with α-tubulin. (e)–(h) Lymph node sections from a viraemic animal stained for either E2 or NS3 and α-tubulin. (e) E2 stained with MAb WB162 and FITC-labelled secondary antibody. (f) Dual staining for E2 with MAb WB162 and FITC-labelled (green) secondary antibody and anti-α-tubulin MAb with CYS-labelled (blue) secondary antibody. White colour indicates co-localization. (g) NS3 stained with MAb WB103 and FITC-labelled secondary antibody. (h) Dual staining for NS3 with MAb WB103 and FITC-labelled (green) secondary antibody and anti-α-tubulin MAb with CYS-labelled (blue) secondary antibody. White colour indicates co-localization.
anti-mouse IgG1 conjugated to FITC (Fig. 2a), and goat antimouse IgG2a conjugated to tetramethylrhodamine isothiocyanate (TRITC) (Fig. 2b). Analysis of confocal images (Leica TCSNT) revealed that in the germinal centres staining positive for E\(^{\text{NS}}\) there was co-localization of E\(^{\text{NS}}\) and immunoglobulin (Fig. 2c). Additional germinal centres containing immunoglobulin deposits were negative for E\(^{\text{NS}}\). The isotype specificities of the second-step antibodies were confirmed by staining sections with different combinations of the first- and second-step reagents. As with single-colour IIF, staining for E\(^{\text{NS}}\) was detected only in infected tissues, whereas a reticular pattern of staining for immunoglobulin was detected in lymph nodes from infected (Fig. 2b) and uninfected (Fig. 2d) animals.

To further confirm whether E\(^{\text{NS}}\) was trapped in extracellular sites in germinal centres, the precise localization of E\(^{\text{NS}}\) was determined by dual staining for E\(^{\text{NS}}\) and tubulin.

Lymph node sections from viraemic animals stained for E\(^{\text{NS}}\) with MAb WB210 and anti-\(\alpha\)-tubulin MAb (clone B-5-1-2; Sigma). The majority of the staining for E\(^{\text{NS}}\) did not co-localize with \(\alpha\)-tubulin (Fig. 1d). This failure to co-localize E\(^{\text{NS}}\) with \(\alpha\)-tubulin is in contrast with the almost complete co-localization of NS3 and E2 with \(\alpha\)-tubulin (Fig. 1f, h).

Sera collected from the six PI calves from which lymph nodes were examined were negative for anti-BVDV antibody as determined by virus neutralization (Brownlie et al., 1984) and ELISA (Howard et al., 1985). In an attempt to detect low levels of antibody in the form of immune complexes in the serum, 7% PEG was added to the serum from one of the calves to precipitate immune complexes (Azazy et al., 1994). Immunoglobulin was purified from the re-dissolved PEG precipitates using a protein G column (Pharmacia). Elution of antibodies from the column and disruption of immune complexes were performed simultaneously using 0·1 M glycine, pH 2·0. The column eluate was immediately neutralized using one tenth volume 1 M Tris, pH 7. The eluted material was examined for the presence of BVDV-specific antibodies by immunoprecipitation of \(^{\text{[35S]}\text{methionine}}\)-labelled proteins from cells infected with cp or ncp Pe515 BVDV, or mock-infected cells (Harlow & Lane, 1988). Sera from a BVDV-hyperimmune animal and an animal that had not been exposed to BVDV were used as control antibodies in the immunoprecipitation assays. No evidence of anti-BVDV antibody was found in the immunoglobulin obtained from PEG precipitates (Fig. 3). However, this does not rule out the possibility that small amounts of antibody of low avidity are being produced.

Given that individual infected cells in lymph node sections stained strongly for NS3, the absence of NS3 staining in those germinal centres that exhibited intense staining for BVDV genome and E\(^{\text{NS}}\) suggests that the virus is not replicating at this site. We can also demonstrate that E\(^{\text{NS}}\) is extracellular and co-localizes with immunoglobulin in the germinal centre, which is known to be in the form of antibody–antigen complexes associated with the surface of FDC. The finding that only a proportion of the germinal centres in 6-week-old PI calves stained positive for E\(^{\text{NS}}\) is also indicative of immune complex deposition rather than a general binding of virus or viral antigen to cells within the germinal centres. The presence of large quantities of viral RNA in the absence of virus replication suggests that the RNA must be protected from degradation, most probably within intact viral capsids. E\(^{\text{NS}}\) has been shown to be associated with the mature BVDV virion and to be produced as free protein (Rümenapf et al., 1993). Whether the E\(^{\text{NS}}\) present in the germinal centres is virus-associated or free could not be determined. However, the presence of large quantities of virus genome suggests that at least some of the E\(^{\text{NS}}\) is associated with virions. Together, these data indicate that BVDV RNA and antigen detected in germinal centres is trapped in immune complexes.

An unexpected finding was the absence of detectable E2 protein in the germinal centre deposits. We also failed to detect E2 in the germinal centre using MAb s that recognize a number of different epitopes on the E2 molecule. Similar findings have been reported by Collins et al. (1999). Antigen localized on the surface of FDC in the form of immune complexes is usually preserved for months in its native conformation. The trapped antigen acts as a long-term reservoir for the induction and
Fig. 3. Radioimmunoprecipitation of BVDV-infected calf testis cells with non-immune (lanes 1, 4 and 7) and hyperimmune (lanes 2, 5 and 8) sera, and serum from a PI animal after PEG precipitation (lanes 3, 6 and 9). Lysates from calf testis cells infected with BVDV Pe515ncp (lanes 4–6), Pe515cp (lanes 7–9) or mock-infected cells (lanes 1–3) were labelled for 4 h with [35S]methionine at 16 h post-infection. Extracts were radioimmunoprecipitated with the test sera and separated by 8% SDS–PAGE. The autoradiograph was developed after 4 days at −70 °C. The molecular masses of the markers are indicated on the left. The most likely identities of the BVDV-specific bands are identified on the right.

maintenance of B cell memory responses (Tew et al., 1997; Bachmann et al., 1996). The possibility that E2 epitopes may be masked by an excess of antibody within the germinal centres is unlikely, given the absence of detectable circulating BVDV-specific antibodies in these animals. A recent study by Weiland et al. (1999) reported that E2 epitopes on BVDV viral particles could not be detected by immunoelectron microscopy when the virions were associated with the cell membrane. Hence, the absence of detectable E2 in germinal centres may be due to conformational alteration upon association with cells in the germinal centres.

Persistent infection with BVDV is established when a calf is infected in utero prior to the onset of immune competence. The absence of circulating antibody and the sustained viraemia in PI calves has been taken as evidence that such animals are immunologically tolerant to the virus. We have also found that PI calves do not give detectable in vitro T cell proliferative responses to the persistent virus (T. Collen and I. Morrison, unpublished data). However, the detection of viral antigen co-localized with immunoglobulin in the majority of the germinal centres in lymph nodes of PI neonatal calves implies that there is a virus-specific B cell response, although careful analysis of serum from the animals failed to reveal any specific circulating antibody. This is in contrast to the study by Collins et al. (1999), who reported the detection, in a proportion of PI animals, of antibody that neutralized the persisting ncp virus. These authors also found evidence of variation in the RNA sequence in two of the animals with circulating antibody, and proposed that changes in B cell epitopes as a result of mutation of the virus were responsible for inducing the antibody response. However, this conclusion is at odds with their observation that serum antibody neutralized the persisting virus, within which the mutant viruses could be expected to represent only a minor component.

The development of fully differentiated germinal centres following trapping of immune complexes by FDC is dependent on specific T cell responses (Liu & Arpin, 1997). The germinal centres identified as containing viral antigen and RNA, in our studies, were not obviously different from germinal centres in normal antigenically stimulated lymphoid tissues, in that they were of similar size, had clearly differentiated light and dark zones and contained small numbers of CD4+ cells. The implication of these findings is that both B and T lymphocytes reactive with the virus are involved. The formation of germinal centres might, therefore, be a consequence of the incomplete immunological tolerance to the virus or result from spontaneous generation of mutations in the virus that give rise to T cell epitopes capable of providing help for differentiation of virus-specific B cells. The latter model assumes that B cell tolerance is incomplete. Incomplete B cell tolerance has been demonstrated in mice infected, either in utero or neonatally, with lymphocytic choriomeningitis virus (Oldstone & Dixon, 1967). These mice had persistently high titres of virus in their blood and no detectable circulating antibody. However, immune complexes could be detected in the kidneys. The absence of detectable BVDV-specific antibody in the serum may be due to consumption of the antibody by an excess of viral antigen in the circulation, although we have failed to detect immune complexes in serum or in the kidneys (data not shown). Alternatively, it is known that B cells in the germinal centres undergo apoptosis and/or are excluded from the germinal centres if exposed to large quantities of soluble viral...
protein (reviewed by Bachmann, 1998). In this way, B cells may be eliminated before they expand to the level where sufficient detectable immunoglobulin is produced in the circulation. The latter situation is potentially similar to that seen in calves persistently infected with BVDV, in which large quantities of viral antigen are present in the blood. We therefore conclude that immune complexes containing E\text{E\textsuperscript{tn}} are present in specific germinal centres of PI animals. We hypothesize that the presence of these immune complexes is due to incomplete B cell tolerance, and that the failure to detect circulating antibodies is the result of failure in maturation of the B cell response coupled with the high load of soluble antigen in these PI animals. More detailed studies of the antigenic specificities of B cells in the lymphoid tissues are required to address this hypothesis.

References


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