Virology **259,** 85–98 (1999)
Article ID viro.1999.9697, available online at http://www.idealibrary.com on IDEAL®

Bovine Viral Diarrhea Virus Quasispecies during Persistent Infection

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Received November 9, 1998; returned to author for revision December 16, 1998; accepted March 10, 1999

Analysis of viral genome sequences from two calves persistently infected with bovine viral diarrhea virus revealed a quasispecies distribution. The sequences encoding the glycoprotein E2 were variable, translating to a number of changes in predicted amino acid sequences. The NS3 region was found to be highly conserved in both animals. The number of E2 clones showing variant amino acids increased with the age of the animal and comparison of the consensus sequences at the different time points confirmed differences in the predicted E2 sequences over time. The immune tolerance that allows the lifelong persistence of this viral infection is highly specific. It is likely that some of the variant viruses generated within these animals will differ antigenically from the persisting virus and be recognized by the immune system. Evidence of an immune response to persisting virus infection was gathered from a larger sample of cattle. Serum neutralizing antibodies were found in 4 of 21 persistently infected animals. Accumulations of viral RNA in the lymph nodes of all animals examined, particularly in the germinal center light zone, may represent antigenic variants held in the form of immune complexes on the processes of follicular dendritic cells.

INTRODUCTION

A fundamental aspect of the pathogenesis of any viral infection is the degree to which different strains can vary in their ability to cause disease. RNA viruses, in particular, can mutate rapidly due to errors introduced during successive rounds of genome replication. Variant viruses or "quasispecies" will be occurring spontaneously within infected cells and the accumulated mutations can influence the growth characteristics of the progeny viruses (Ahmed and Oldstone, 1988; Banner and Lai, 1991; Dockter *et al.,* 1996; Domingo and Holland, 1997; Leister *et al.,* 1993). Where mutations enhance the survival of a particular genome sequence, rapid expansion of clones derived from this source may subsequently come to dominate the resulting viral population (Domingo and Holland, 1997; Enomoto and Sato, 1995). That this population dynamic is operating is well documented for many persistent or chronic viral infections, including human immunodeficiency virus (HIV) and hepatitis C virus (HCV), where the selection pressures driving viral evolution may include the host immune response to infection or antiviral therapy (Enomoto and Sato, 1995). One exception to this scenario was thought to be the persistent infection of cattle with bovine viral diarrhea virus (BVDV), where the unique immunotolerance of the host for the persisting viral infection was considered to favor the maintenance rather than the antigenic evolution of the viral population (Hertig *et al.,* 1995; Paton *et al.,* 1994).

BVDV is a positive sense, single-stranded RNA virus, classified within the *Pestivirus* genus in the family Flaviviridae (Westaway *et al.,* 1985). There are two biotypes of the virus which are distinguished by their ability to cause cytopathology in cell culture (Baker *et al.,* 1954; Brownlie *et al.,* 1984; Gillespie *et al.,* 1960; Underdahl *et al.,* 1957). The predominant, non-cytopathogenic biotype (BVDVnc) is found in both acute and persistent infections in cattle and is associated with a wide range of clinical signs. Persistent infections arise following fetal infection *in utero* during the first trimester; as the fetus develops the virus is not recognized by the immune system, with a consequent lack of immune response to the persisting virus (Bolin *et al.,* 1985a; Brownlie *et al.,* 1984). These persistently infected (p.i.) animals are, however, capable of responding normally to other antigens and to acute infections with different BVDV isolates producing a normal neutralizing antibody response (Bolin *et al.,* 1985b; McClurkin *et al.,* 1984; Steck *et al.,* 1980; Westenbrink *et al.,* 1989).

The BVDVnc genome is approximately 12.5 kb in size (Deng and Brock, 1992) and, in common with many other RNA viruses, readily demonstrates recombination as a result of template switching during replication (Desport *et al.,* 1998; Meyers *et al.,* 1992; Qi *et al.,* 1992). This process can give rise to the second, cytopathogenic biotype of the virus (BVDVc) which only occurs in p.i. cattle and precipitates the death of the animal from

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession Nos. AJ241224 to AJ241252 inclusive.

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0042-6822/99 $30.00

a Alignment of the sample consensus sequences from all samples

```
          1                                                                                                100
   302928                                                                                          E
   309938                                    N                                            K        E
   904928                                                                                          G
   903938                    L                                                                     G
Consensus HLDCKPEYSYAIAKNDRIGPLGAEGLTTVWKDYSPEMTLEDTMVIASCRDGKFMYLSRCTRETRYLAILHSRALPTSVVFEKLFEGQKQ-DTVEMDDNFE

          101                                                                                              200
   302928
   309938
   904928
   903938
Consensus FGLCPCDAKPIVRGKYNTTLLNGPAFQMVCPIGWTGTVSCMLANRDTLDTAVVRTYRRSRPFPYRQDCITQKVLGEDLYDCILGGNWTCITGDQLQYSGG

          201                    229
   302928
   309938          Y
   904928
   903938              T
Consensus SIESCKWCGFKFQRSEGLPHYPIGKCRLK
```

FIG. 1. Aligned translated protein sequences from the BVDV E2 amplimers derived from persistently infected animals 1263 and 1279. The consensus sequences obtained by direct PCR sequencing of amplified viral RNA (two samples for each animal at two different time points) are aligned in (a). Alignment of sequences derived from individual cloned PCR products is shown in (b) to (e). The consensus sequence is displayed as the lower line and changes from the consensus (in individual PCR clones) are displayed above this sequence. Agreement with the consensus is not displayed.

mucosal disease (MD). BVDVc genomes have been reported to vary in size from a defective interfering particle of 7 kb (Kupfermann *et al.,* 1996; Tautz *et al.,* 1994) to genomes of over 16 kb (Meyers *et al.,* 1992), arising by deletion or duplication of portions of the genome, by point mutations, or by recombination with host cellular mRNAs (reviewed in Meyers and Thiel, 1996). As the BVDVc arises by mutation of the BVDVnc within a p.i. animal, the antigenic homology between the pairs of viruses that cause MD is high (Corapi *et al.,* 1988; Howard *et al.,* 1987). Thus the complex pathogenesis of fatal MD in cattle is intimately related to the specificity of immunological tolerance engendered as a consequence of early fetal infection.

The highest degree of variability between different BVDVnc strains occurs in the major glycoprotein (E2) sequences and neutralizing antibodies to this protein provide complete protection from infection (Deng and Brock, 1992; Howard *et al.,* 1989). In a p.i. animal it was assumed that the specificity of the immunological tolerance of the host favored maintenance of an antigenically conserved population of the persisting virus over the emergence of quasispecies which stimulate an effective immune response. Thus although RNA sequence variants may arise, those which were recognized as antigenically heterologous by the immune system were thought to be rapidly cleared. Analysis of E2 sequences obtained from the serum of p.i. animals at different time points apparently confirmed that the persisting virus sequence was maintained in stasis, with two groups independently showing no variation in the consensus nucleotide sequence of E2 within a p.i. animal (Hertig *et al.,* 1995; Paton *et al.,* 1994). Although the stability of the overall virus population was not unexpected, the absolute conservation of RNA sequence over time, without any silent nucleotide substitutions, was surprising given the heterogeneity of E2 sequences between different virus strains and the known properties of viral RNA polymerases.

The aims of our study were (i) to determine whether RNA sequence variants arise during persistent BVDV infections and (ii) to seek evidence of an immune response to the persisting virus. In a retrospective analysis, samples from persistently infected animals were used as a source of viral RNA to identify potential sequence variations and tested for the presence of neutralizing antibody. Lymphoid tissues from a total of 21 experimental or field cases of persistent BVDV infection were examined by *in situ* hybridization and by immunocytochemistry using monoclonal antibodies to differentiate between actively replicating virus and assembled virions.

RESULTS

Analysis of virus sequence variation from animals 1263 and 1279

The E2 region of BVDVnc was amplified from total RNA extracted from two persistently infected calves (1263 and 1279) and two different time points (taken at 11- and 18-month intervals) for each animal (giving sample data 302928 = 1263 early, 309938 = 1263 late, 904928 = 1279 early, 903938 = 1279 late). The PCR products (generated using the enzyme *Pfu* polymerase to minimise the frequency of *in vitro* induced errors) were sequenced directly to determine the consensus sequence of the viral population present in the sample. A small number of differences are apparent in the alignment of the consensus protein sequences (Fig. 1a).

The clonal composition of the virus populations at

b 1263 early

```
          1                                                                                                  100
 302928a                                                  S                          K
 302928b
 302928c                                                                             K
 302928d                                                                             K
 302928e
 302928f
Consensus HLDCKPEYSYAIAKNDRIGPLGAEGLTTVWKDYSPEMTLEDTMVIASCRDGKFMYLSRCTRETRYLAILHSRALPTSVVFEKLFEGQKQEDTVEMDDNFE

          101                                                                                                200
 302928a                                                                                  M
 302928b
 302928c
 302928d                                                        H
 302928e
 302928f
Consensus FGLCPCDAKPIVRGKYNTTLLNGPAFQMVCPIGWTGTVSCMLANRDTLDTAVVRTYRRSRPFPYRQDCITQKVLGEDLYDCILGGNWTCITGDQLQYSGG

          201                       229
 302928a
 302928b
 302928c
 302928d
 302928e
 302928f
Consensus SIESCKWCGFKFQRSEGLPHYPIGKCRLK
```

c 1263 late

```
          1                                                                                                  100
 309938a                                  N    E
 309938b                                  N
 309938c
 309938d                                                        A
 309938e                                      K
 309938f
Consensus HLDCKPEYSYAIAKNDRIGPLGAEGLTTVWKDYSPNMTLEDTMVIASCRDGKFMYLSRCTRETRYLAILHSRALPTSVVFKKLFEGQKQEDTVEMDDNFE

          101                                                                                                200
 309938a                                      M
 309938b
 309938c
 309938d
 309938e
 309938f
Consensus FGLCPCDAKPIVRGKYNTTLLNGPAFQMVCPIGWTGTVSCMLANRDTLDTAVVRTYRRSRPFPYRQDCITQKVLGEDLYDCILGGNWTCITGDQLQYSGG

          201                       229
 309938a
 309938b
 309938c
 309938d
 309938e
 309938f
Consensus SIESCKWCGYKFQRSEGLPHYPIGKCRLK
```

FIG. 1—*Continued*

each time point was investigated by cloning the PCR products in *Escherichia coli.* Individual recombinant plasmids were sequenced in both directions and an unambiguous sequence was obtained for each clone. These sequences were aligned using the GCG package of programs to highlight differences in the E2 sequences within an animal. A summary of the sequence changes occurring is shown in Table 1, while protein translation alignments are shown in Figs. 1b and 1c (animal 1263) and Figs. 1d and 1e (animal 1279). The individual sequences from each of the E2 clones showed variation with respect to other clones and to the consensus sequence for that sample.

To exclude the possibility that some of these sequence changes occurred *in vitro* during the RT/PCR process, clones were generated from the highly conserved NS3 region of the viral genome. A total of 10 clones were analyzed from the two animals and sequence alignment revealed a much lower variability between both clones and animals. The consensus nucleotide sequences for both 1263 and 1279 were identical. Of the 4 nucleotide changes observed, only 1 resulted in an amino acid substitution (Fig. 2). The NS3 amplimers were shorter than the E2 amplimers examined so, to prevent any bias, an analysis of the percentage of variation from the consensus per nucleotide sequenced was undertaken. The variation of 0.49% (37 nucleotide changes/7557 bases sequenced) for the E2 sequences was more

d 1279 early

```
         1                                                                                                  100
 904928a
 904928b
 904928c                     L                                                                          E
 904928d                     L                                                                          E
 904928e
 904928f                                                                                    K          E
 904928g
 904928h                     L                                                                          E
Consensus HLDCKPEYSYAIAKNDRIGPLGAEGLTTVWKDYSPEMTLEDTMVIASCRDGKFMYLSRCTRETRYLAILHSRALPTSVVFEKLFEGQKQGDTVEMDDNFE

         101                                                                                                200
 904928a
 904928b
 904928c                       S
 904928d                       S
 904928e
 904928f                       S
 904928g                                                 I
 904928h                       S
Consensus FGLCPCDAKPIVRGKYNTTLLNGPAFQMVCPIGWTGTVSCMLANRDTLDTAVVRTYRRSRPFPYRQDCITQKVLGEDLYDCILGGNWTCITGDQLQYSGG

         201                       229
 904928a               G
 904928b               S
 904928c
 904928d
 904928e               G
 904928f
 904928g                R
 904928h                R
Consensus SIESCKWCGFKFQRSEGLPHYPIGKCRLK
```

e 1279 late

```
         1                                                                                                  100
 903938a
 903938b
 903938c N                   P                         T           P                    K          E
 903938d                                                             R                P
 903938e
Consensus HLDCKPEYSYAIAKNDRIGLLGAEGLTTVWKDYSPEMTLEDTMVIASCRDGKFMYLSRCTRETRYLAILHSRALPTSVVFEKLFEGQKQGDTVEMDDNFE

         101                                                                                                200
 903938a
 903938b
 903938c                       S                                                          T
 903938d
 903938e                                                                                H
Consensus FGLCPCDAKPIVRGKYNTTLLNGPAFQMVCPIGWTGTVSCMLANRDTLDTAVVRTYRRSRPFPYRQDCITQKVLGEDLYDCILGGNWTCITGDQLQYSGG

         201                       229
 903938a
 903938b
 903938c               RR              N
 903938d                         L
 903938e
Consensus SIESCKWCGFKFQTSEGLPHYPIGKCRLK
```

FIG. 1—*Continued*

than five times greater than the 0.09% (4 nucleotide changes/4230 bases sequenced) for the NS3 sequences.

Further analysis of the E2 nucleotide sequences from the earliest samples for both animals, 1263 and 1279, shows variation between individual clones within a sample. Clonal differences from the sample consensus sequence were observed at between 9 and 13 positions, translating to amino acid variation at 4 and 7 positions in the E2 protein sequences. The observed variation constituted either a single difference in a single clone or more equal mixtures of two or more virus populations. The original virus inoculum used to infect both animals was not available for examination but it is possible that such mixed populations reflect sequence variation in the initial challenge dose. Comparison of these sequence data with samples taken some months later is more revealing (Figs. 1b to 1e).

The E2 clones from the later time points sampled show, for calf 1263, nucleotide changes resulting in clonal variation occurring at 11 positions over the 687 nucleotides sequenced (4 amino acid differences), with the result that none of the clones showed 100% homology with respect to each other. The sequence variation in clones from 1279 was even greater, with variation at 18 positions (resulting in 15 amino acid differences). Align-

TABLE 1

Summary Analysis of the Consensus Sequences Derived from the E2 Region of BVDVnc Amplified (687 Bases) from Total RNA of Two Persistently Infected Calves (1263 and 1279) and Two Different Time Points

	No. of positions of variation	Transitions	Transversions	No. of amino acid changes
302928	9	7	2	4
309938	11	9	2	4
904928	13	9	4	7
903938	18	11	7	15

Note. Sample data: 302928 = 1263 early, 309938 = 1263 late, 904928 = 1279 early, 903938 = 1279 late.

ment of the clonal sequences suggests an homogenous population change within samples at a limited number of points but this must always be qualified by the limited number of individual clones analyzed from that sample. However, alignment of the sample consensus sequences (Fig. 1a) confirms changes to the nucleic acid and also the consensus protein sequences in different samples at different times. The consequence of this variation is a small change in the consensus translated amino acid sequence for E2 within an animal over time.

Further analysis of the variant E2 sequences from both animals 1263 and 1279 using the PHYLIP package of programs estimated the phylogeny of the clonal sequences using the maximum likelihood analysis program, DNAML4. Figure 3 illustrates the evolutionary relationships of these virus sequences as displayed by the program, DRAWGRAM and confirms, with >97% confidence, the clustering of sequences derived from each of the two different animal sources.

Serum neutralization tests

Serum samples from 21 persistently infected animals (including 1263 and 1279) were examined for the presence of virus neutralizing antibodies. Four samples were positive in this assay when tested against the NADL reference strain (Table 2). To try to establish whether these antibodies were induced by the endogenous, persisting virus or as a result of exposure to a heterologous strain of BVDV, virus purified from serum samples of animals 1263 and 1279 was used in an homologous neutralization assay. The neutralization titer to the homologous virus was higher than that to the NADL strain.

Immunocytochemistry

The distribution of BVDV-specific antigen during persistent infections was investigated using cryopreserved prescapular lymph nodes from calves 1263 and 1279. Monoclonal antibodies specific for the NS2/3 nonstructural protein and the E2 and E^{rns} envelope glycoproteins were used to discriminate between intracellular and potentially extracellular accumulations of the virus. Cells supporting replication of BVDVnc, staining with antibody WB103 (specific for NS2/3), showed a widespread distribution throughout the lymph node (Fig. 4c). The distribution of the E2 glycoprotein (antibody WB214) was similarly widespread (Fig. 4a). In contrast, the distribution of the E^{rns} glycoprotein (antibody WB210) was mainly confined to the germinal centers of the lymphoid follicles (Fig. 4b). Some germinal centers (GC) had a distinct crescent-shaped area of staining corresponding to the light zone. The E^{rns} protein is known to be both virion associated and secreted from infected cells; the staining observed here could be attributable to either form of this protein.

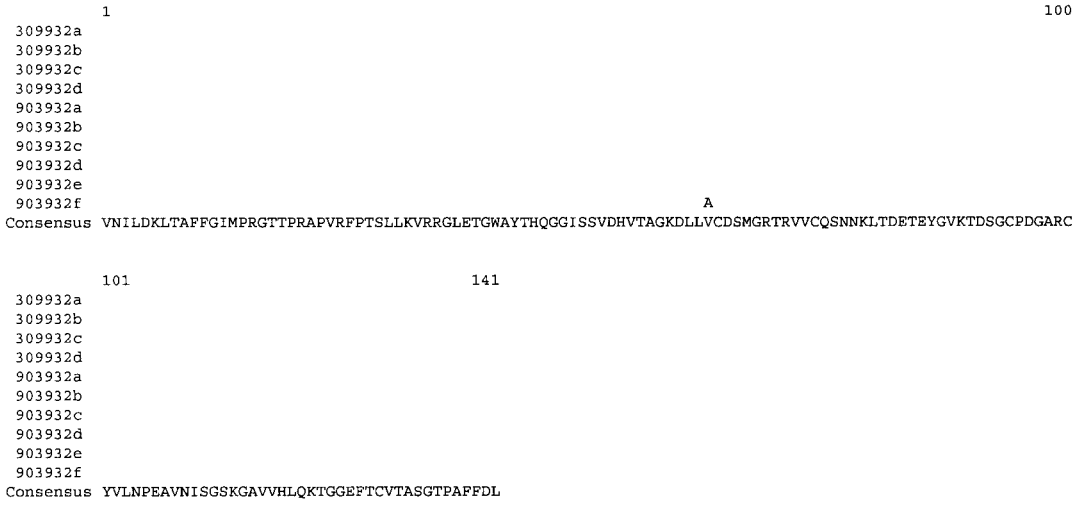

FIG. 2. Aligned translated protein sequences from the NS3-derived amplimers from animals 1263 and 1279. The consensus sequence is displayed as the lower line and changes from the consensus are displayed above this sequence. Agreement with the consensus is not displayed.

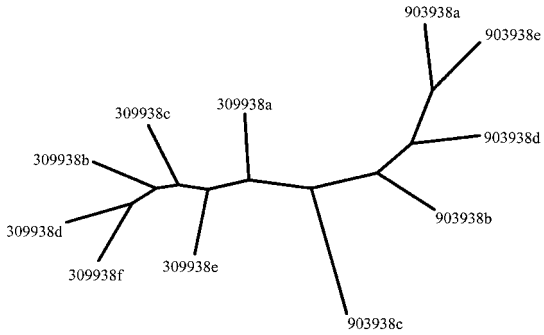

FIG. 3. Estimation of the phylogeny of the individual clonal E2 sequences amplified from the sera of animals 1263 and 1279 using the maximum likelihood method of the program DNAML4. Bootstrap analysis with 100 repetitions confirmed the significance (>97%) of the clustering shown.

In situ hybridization

Lymph node sections from 21 persistently infected animals were examined for the distribution of BVDV RNA using *in situ* hybridization (ISH) with a riboprobe specific for the NS2/3 region of the virus genome (see Fig. 4e and Table 2). Viral RNA was detected throughout the sections but additionally, intense GC staining for the BVDV positive strand RNA was found to varying degrees in all of the cases examined by ISH (Fig. 4e). There seemed to be a reciprocal balance between the generalized staining of T-cell areas and intense staining of GCs; animals with the highest levels of RNA-positive GCs had the lowest levels of perifollicular staining and vice versa. As reported in an earlier study (Desport *et al.,* 1994), these accumulations of BVDV RNA are found in many of the germinal centers that are also positive by immunocytochemistry for E^{rns} but do not reflect increased viral replication, as staining for the NS2/3 protein never shows similar variation in density.

The pattern of ISH staining could be used to group the animals into three categories with respect to GC staining: high (+++), BVDVnc RNA was detected in 35–60% of GCs with little or no staining in the T-cell areas; medium (++), BVDVnc RNA in 5–35% of the germinal centers, with a moderate degree of RNA-positive cells staining in the T-cell areas of the node and in the ducts; or low (+), less than 5% BVDV RNA-positive GCs but significant staining in the T-cell areas of the node (Table 2).

The four animals identified as having neutralizing antibody in their sera had high levels of germinal center RNA staining. The animals with fewer than 5% RNA-positive germinal centers were mainly the youngest cases and the majority of these had a high level of

TABLE 2

Twenty-One Persistently Infected Cattle Were Screened for the Presence of Serum Neutralizing Antibodies to BVDV (Strain NADL) and for the Presence of Virus in Prescapular Lymph Nodes Using Both Monoclonal Antibodies and a BVDV-Specific Riboprobe

	Animal identity no.	Age	Level of GC staining	T cell area staining	Neutralising antibody titre
1	0232	5 months	+++	−	+1/16
2	1216	4 months	+	−	Not detected
3	3382	2 months	+	+	Not detected
4	3452	2 months	+	+	Not detected
5	1263	7 months	+++	−	+1/16
6	1279	8 months	+++	−	+1/32
7	1280	2 months	+	++	Not detected
8	2342	7 months	+	+++	Not detected
9	2350	7 months	+++	−	Not detected
10	2397	8 months	+++	−	+1/128
11	2401	5 months	+	+++	Not detected
12	DP197	N/A	++	−	Not detected
13	Shirley 2HS	N/A	+++	−	Not detected
14	VF 2315	N/A	++	+	toxic < 1/24
15	0825	33 months	+++	−	Not detected
16	1035	24 months	++	−	Not detected
17	1409	16–20 months	++	+	Not detected
18	1448	16–20 months	+	++	Not detected
19	59B	2 months	++	+	Not detected
20	62B	2 months	++	+++	Not detected
21	63B	2 months	+	+++	Not detected

Note. The level of germinal center (GC) staining by *in situ* hybridization was scored as +++/high (35–60% of GCs), ++/medium (5–35% of GCs), or +/low (less than 5% positive GCs). The ISH staining pattern was very similar to the staining pattern of antibody WB210. The degree of T-cell area staining reflects the distribution of NS3 antigen detected by antibody WB103. The neutralizing antibody titer is the dilution of serum which gave a 50% inhibition of virus production.

generalized virus-positive staining throughout the T-cell areas of the node.

DISCUSSION

The aim of this study was to seek evidence for the existence of quasispecies of BVDV within persistently infected cattle. The presence of RNA virus quasispecies during long-term or persistent infections is widely accepted for many diseases and is thought to contribute to the pathogenic mechanisms of the virus—random variants arise through the infidelity of the viral RNA polymerase and may be selected to become the dominant viral population as a result of increased replicative fitness (Domingo *et al.,* 1996; Domingo and Holland, 1997; Enomoto and Sato, 1995). Consequently, analysis of the viral population in an infected individual at different time points will confirm the constant mutation of the viral genome sequence. In contrast, it has been suggested that the specific immunotolerance generated by *in utero* infection of cattle with BVDVnc permits the survival of the persisting virus while maintaining the immune competence required to clear infection with heterologous BVDVnc strains. While it is obvious that immune recognition or escape is only one of many selective pressures influencing the evolution of the viral population within an infected animal, this situation might be expected to favor the persisting virus and eliminate antigenic variants or quasispecies arising during viral replication. In support of this, two reports (Hertig *et al.,* 1995; Paton *et al.,* 1994) have reported that consensus E2 sequences in serum samples taken from a single animal were 100% identical, even over a 1-year sampling period. However, such consensus sequences can mask significant variation in the component virus populations and it is possible that variants with increased replicative fitness could survive within these populations. In order to determine the significance of quasispecies in a BVDV-infected animal, we analyzed both consensus sequences and individual PCR fragments isolated from two p.i. calves experimentally infected *in utero* with the Pe515nc strain of BVDV. The consensus sequences of the region encoding the major viral glycoprotein, E2, varied in both RNA and translated amino acid sequence, an observation in contrast with previously published data. At one point (Fig. 1a, amino acid position 90) the consensus protein sequence was represented by E in both samples from animal 1263 and by G in 1279. At other points the consensus of the early sample differed from the later sample taken from the same animal. As the same initial inoculum was used to infect both fetuses (not available for analysis), these data, if taken in isolation, could reflect different possibilities. Either the same pool of viruses could have established infection in each of the animals examined (1263 and 1279) or distinct viral variants (present in the inoculum) established persistent infection in each host animal. Subsequently, the starting virus populations may have been maintained in genetic stasis as a consequence of the immunotolerance or evolved slowly and independently in response to other pressures on virus survival. However, the observation that the consensus viral sequence obtained from both animals varied with time confirms a dynamic process of viral evolution.

As the expectation was for any sequence variation occurring in the persisting virus populations to be at low frequency, it was essential that the possibility of introducing bias due to infidelity during RT–PCR was minimized. The enzyme *Pfu* polymerase was chosen for amplification because of the low error rate (almost error free, Bracho *et al.,* 1998) and was further supported by the results of a control amplification of the viral genome region encoding the NS3 protein. When the NS3 amplimers were sequenced, 7 of the 10 clones from both animals had 100% identity; single nucleotide changes were observed in 3 NS3 clones, only one change resulted in an amino acid substitution. This conservation of NS3 sequences is in stark contrast to the results obtained for E2. Again, Bracho *et al.* (1998) conclude that the error rate of *Taq* polymerase was "not significantly affected by sequence context" (although no comment was possible about *Pfu* polymerase as the induced error rate was too low). This further suggests that the differences in observed error rates for the E2 and NS3 sequences are neither enzyme-induced nor sequence context-dependent but confirm that the variations observed in E2 sequences are real and may reflect different selective pressures affecting the different genome regions.

Analysis of sequence variation in the E2 coding region of clones from later time points confirmed that there had been significant changes in the RNA and translated amino acid sequences from both animals over time. For animal 1279, the interclonal variation increased with age; amino acid variation was observed at 7 positions in the earlier samples, increasing to 15 variant positions in the later sample. It is striking to observe that 15 of 18 cDNA sequence changes were predicted to result in protein sequence changes in these samples. More importantly, the consensus sequences of the virus populations sampled (by direct PCR sequencing) differed at three positions between the early and late samples for 1263 and at 2 positions for 1279. This small but possibly significant change does not reflect genetic stasis of the component clonal populations but, in contrast to previously published data, demonstrates the evolution of viral quasispecies within the persistently infected host. The biological significance of these changes still requires elucidation.

Although the number of sequence changes was still relatively small, phylogenetic analysis of the variants using the program DNAML4 was able to show the relationship between the different clones and group together sequences derived from either 1263 or 1279. This again

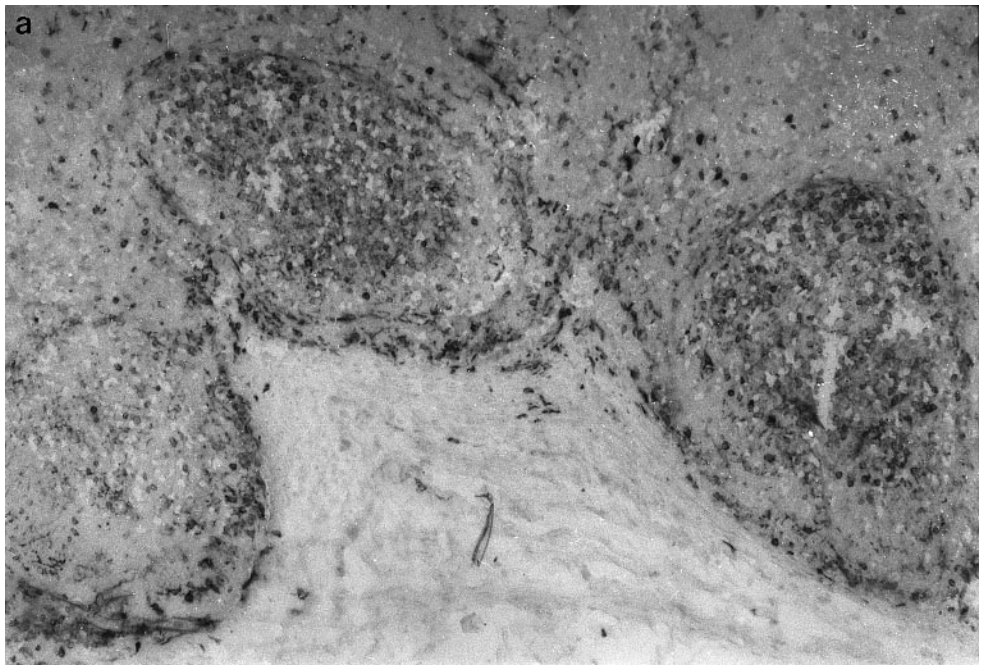

FIG. 4. Serial sections (a–d) from the prescapular lymph node of persistently infected calf 1279 using monoclonal antibodies to detect BVDV antigens: (a) WB214 to detect distribution of structural glycoprotein E2, (b) WB210 to detect structural glycoprotein E^{ms}, and (c) WB103 to detect nonstructural protein NS2-3. (d) TRT 1 negative control and (e) *in situ* hybridization to detect BVDV positive sense RNA. The distribution of cells supporting virus replication is indicated in (c). Differences in the detection of other viral components, particularly the E^{ms} protein and genomic RNA (in b and e), may reflect the trapping of immune complexes on follicular dendritic cells.

suggests the evolution of related virus sequences within an animal rather than the maintenance in stasis of an initial population of viruses selected from a common pool in the inoculum. Bootstrap analysis with 100 repetitions confirmed the significance (>97%) of the clustering shown.

The data indicate an increase in amino acid sequence variation of the component virus populations over time that has the potential to generate antigenically variant viruses. The epitopes involved in the antigenicity of E2 have not yet been precisely defined for BVDV, although antigenic regions have been determined for CSFV (Van Rijn *et al.,* 1994). The amino acid changes observed were in the E2 regions equivalent to domains B and C but not in A or D (Van Rijn *et al.,* 1994). Regions of hypervariability have been outlined for BVDV by both sequence comparisons and by the generation of neutralization escape mutants. The change from I to T (at position 182) in clone 903938c (this study) corresponds exactly with an escape mutant generated by Paton (Paton *et al.,* 1992) and thus potentially represents a neutralization escape mutant generated *in vivo*. In the context of an immunotolerant p.i. animal, this may represent the mutation of an epitope from one which is not recognized by the host to one which becomes the target for immune clearance. Further evidence for the presence of an antibody response to the persisting virus was then sought.

Data were collected from 21 p.i. cattle covering a range of ages and histories (including gnotobiotically derived calves, animals resulting from experimental infection of the dam but subsequently housed in isolation, and also a number of persistently infected animals collected from farm studies). The presence of low levels of neutralizing antibody was confirmed in 4 animals. In all cases these animals had been held in isolation conditions, some had been housed with animals from the same cohort, p.i. with the same virus, but other cohort members remained antibody negative. Neutralizing antibodies have been described in p.i. animals (Duffell and Harkness, 1985; Steck *et al.,* 1980) and this has largely been assumed to be due to infection with heterologous BVDV isolates. Further analysis of these sera confirmed that, for two isolates in the current study, the neutralizing antibody titer to the homologous virus (isolated from serum) was higher than that to the standard NADL reference strain normally used for these assays. As more than a single serum sample was available for each of these animals, the levels of antibody could be followed over time and strongly suggested that the antibody response reflects seroconversion to antigenic variants within the persisting virus population rather than to challenge with a heterologous virus isolate. This surprising conclusion is not unique. Observations made by Edwards *et al.* (1991) and Brock *et al.* (1998) confirm that some cattle maintain a state of persistent viremia in the face of a cycling neutralizing antibody directed toward the homologous virus infection. These observations are consistent with the endogenous recognition of antigeni-

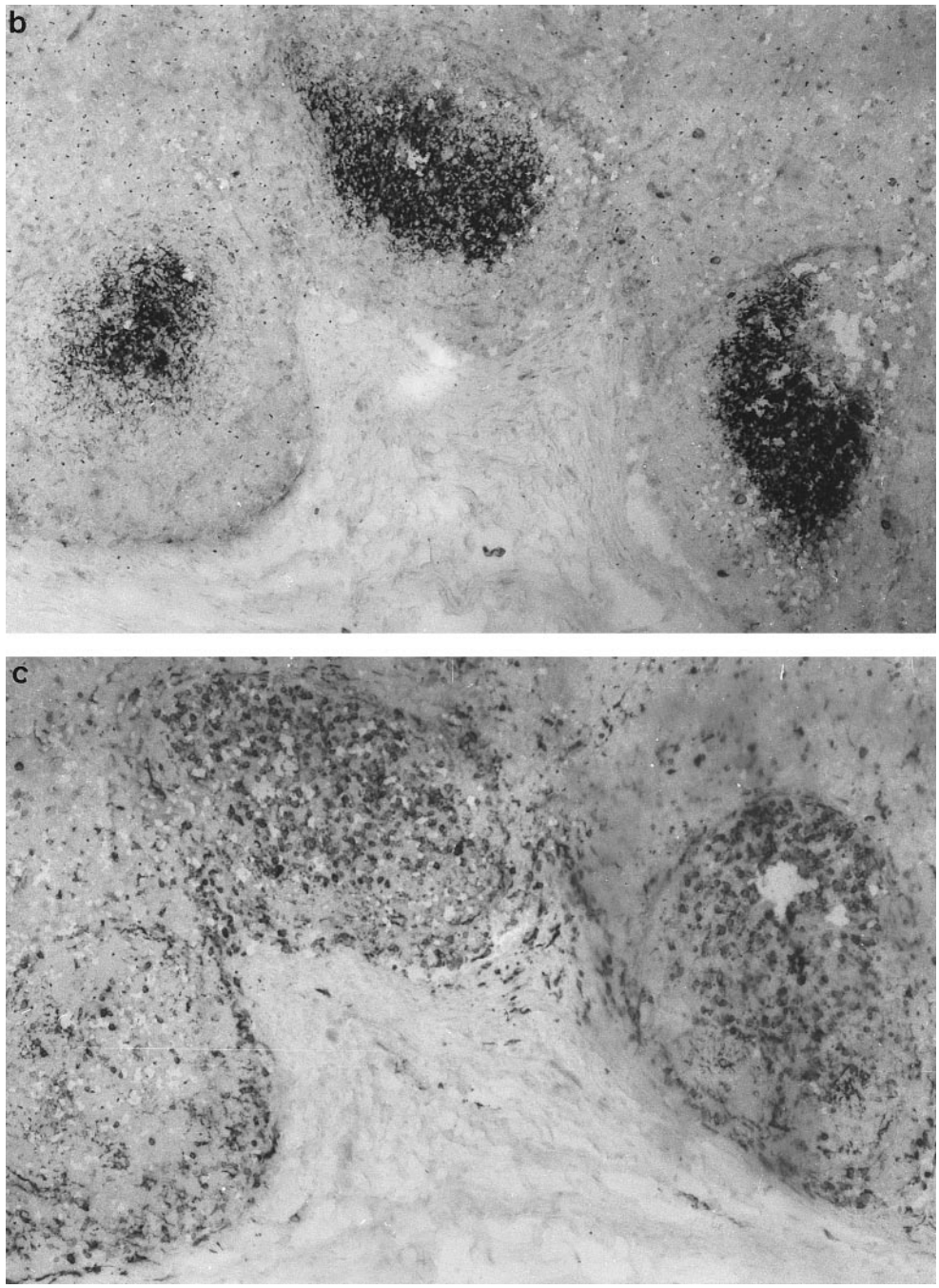

FIG. 4—*Continued*

cally variant viruses and either partial clearance followed by recrudescence or serial emergence and recognition of several independent variants. The alternative that animals held in isolation in three different laboratories had repeated accidental challenge with exogenous heterologous viruses seems less likely. It is possible that some other defect in the immune competence of these animals prevents clearance of the infection.

The presence of a low-level neutralizing antibody response to components of a persisting virus infection may be expected to reflect some aspects of autoimmunity. In general, small immune complexes are formed when an individual makes antibodies to self-antigens, as only a few epitopes are recognized and the formation of a cross-linking lattice is restricted (Roitt *et al.,* 1993). The size of the aggregate influences the site of immune complex deposition—relatively large complexes are deposited in kidneys (such immune complex disease is rarely associated with BVDV infection (Hewicker *et al.,* 1987)), whereas smaller complexes may pass through the glomerular basement membrane. The coexistence of antiviral antibody and antigen in a p.i. animal is likely to

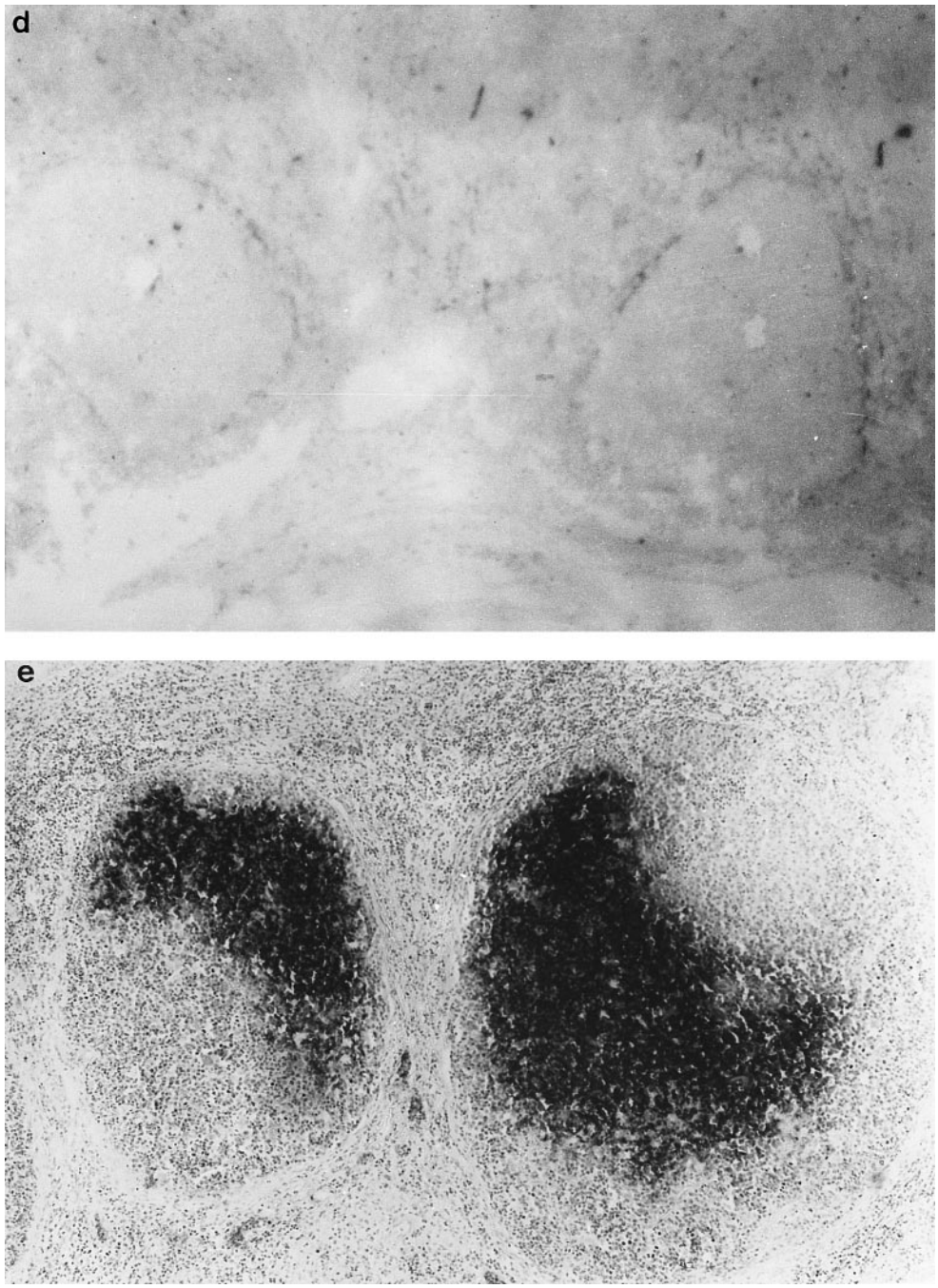

FIG. 4—*Continued*

form small circulating immune complexes that would be trapped and held on the processes of follicular dendritic cells within the germinal centers of peripheral lymph nodes, where they would persist for long periods of time (Roitt *et al.,* 1993). The presence of small immune complexes in serum is difficult to detect and, in this retrospective analysis, the freeze–thawing of samples would have disrupted such complexes. Enduring evidence for their existence in p.i. animals was sought in examination of fixed lymph node sections.

During persistent infection BVDVnc has a wide-ranging tropism, with many different cell types, including most of the peripheral blood mononuclear cells, showing some degree of infection (Bielefeldt-Ohmann, 1995; Sopp *et al.,* 1994). Particularly high levels of virus occur in lymphoid tissues, where a widespread distribution of virus, in both T- and B-cell areas, is observed (Figs. 4a and 4c, Lopez *et al.,* 1993; Sopp *et al.,* 1994). The use of monoclonal antibodies to the viral NS2/3 protein confirmed the active replication of virus within these cells (Fig. 4c and Sopp *et al.,* 1994). In contrast to these data, detection of viral RNA by *in situ* hybridization consistently

indicated intense accumulations of BVDV in the GC areas of the lymphoid follicles (Fig. 4e) in addition to a more even background distribution. There is no evidence to suggest that germinal center B-cells are preferentially infected with BVDVnc; the generalized distribution of the virus NS2/3 protein (Fig. 4c) indicates the contrary. However, the contrasting data generated by different detection reagents (compare Figs. 4a and 4c with 4b and 4e) requires explanation.

Several viruses have been described within GCs either because the cells that populate these areas are specific targets for virus replication (Bachmann *et al.,* 1996; Hufert *et al.,* 1997) or because virus-specific immune complexes are held as iccosomes on the cytoplasmic processes of the follicular dendritic cells (Tew *et al.,* 1997). It is well established that immune complexes can be held in this location as dense aggregates of antigen and RNA, in an undegraded and potentially infectious state, for several months (Bachmann *et al.,* 1996; MacLennan, 1994; Tacchetti *et al.,* 1997). The concentration of positive strand viral RNA (Fig. 4e) and the viral E^{rns} protein (Fig. 4b) at the GC sites would be consistent with the trapping of virus particles from the circulation in small immune complexes. The absence of a similar staining pattern with a monoclonal antibody specific for the E2 protein could be explained if the epitopes on the more variable E2 protein were already blocked by endogenous bovine antibody. This would also be consistent with the absence of an intense GC staining signal with the NS2/3-specific monoclonal, as there would be little or no replicating virus (and therefore no NS2/3 protein) in the trapped immune complexes.

The observations described in this report, including the detection of viral sequence variants, changes in the consensus sequence over time, antibodies to the persisting virus, germinal centers staining strongly for virion components and for complement C3c (unpublished data), are all consistent with the hypothesis that RNA quasispecies arising within a p.i. animal stimulate an endogenous antiviral immune response in the immunotolerant animal. The number of viral variants within an animal will change over time and will certainly be different between animals. The sequestration of the immune complexes generated, into the secondary lymphoid tissues, may contribute to the perception that lymphoid tissues are a preferred site of virus replication and may also exacerbate perturbations of the immune competence of these animals.

Relatively little is known about the effects of variation in the E2 sequence of different BVDV isolates on viral pathogenesis. In other viral infections, such as lymphocytic choriomeningitis virus, a single amino acid change is sufficient to dramatically alter the tropism of the virus (Teng *et al.,* 1996). Current understanding of the structure of the BVDV E2 protein and its interaction with cellular receptor(s) and mechanisms of virulence are too limited to permit speculation about the molecular events occurring during infection, but variation in E2 sequences could influence the virulence of different strains.

MATERIALS AND METHODS

Virus and animals

The Pe515nc strain of BVDV was a virologically cloned non-cytopathogenic virus isolated from a cow diagnosed with mucosal disease. Virus isolation in calf testis (CTe) cells was followed by expansion of the virus stock. All experimental inocula were prepared from infected CTe cells, three passes from the initial virus cloning. Two persistently infected calves, 1279 and 1263, were the offspring of two seronegative dams that had been infected intranasally with Pe51nc before 90 days of gestation. The p.i. calves were held in a secure unit with other animals infected with the same virus. Number 1154 was a normal uninfected calf of a similar age but housed separately from the p.i. animals. Prescapular lymph nodes and serum samples were taken at a number of time points, the first at approximately 7 months of age (samples 1263 early and 1279 early), the latest at 18 months (sample 1279 late) or 21 months of age (sample 1263 late). Samples were cryopreserved at −70°C or fixed in neutral buffered formalin until required.

Lymphoid tissues from an additional 19 experimental or field cases of persistent BVDV infection (detailed in Table 2) were also examined. Tissues, fixed for 24–72 h in neutral buffered formalin, were processed to paraffin blocks prior to use in immunocytochemistry and *in situ* hybridisation.

RNA extraction

RNA was isolated from serum or tissue samples of persistently infected animals using RNA-Stat 60 (AMS Biotechnology) according to the manufacturer's instructions. Briefly, 1.5-ml aliquots of serum were mixed with 15 ml of RNA-Stat 60 and 3 ml of chloroform. The total RNA was separated from the DNA and proteins by centrifugation at 12,000 *g* for 10 min. The colorless upper aqueous phase was removed to a fresh tube and the RNA was precipitated with a total of 7.5 ml of isopropanol. Glycogen was added to assist with the recovery of the RNA pellet. The pellet was obtained after centrifugation as before and initially resuspended in 300 μl diethyl pyrocarbonate (DEPC)-treated H_2O. The RNA was reprecipitated with 12 μl 5 M NaCl and 600 μl ethanol and centrifuged as before. The pellet was washed with 1 ml 75% ethanol before finally resuspending in 50 μl DEPC-treated H_2O.

RT/PCR, cloning, and sequencing

A 5-μl sample of the total RNA was reverse transcribed using random hexamers and 200 units of Super-

Script II Reverse Transcriptase (Gibco/BRL) for 50 min at 42°C in a total volume of 20 μl according to the manufacturer's instructions. Cloned *Pfu* DNA polymerase was used for the PCR as it has a higher fidelity for DNA synthesis than *Taq* polymerase (Bracho *et al.,* 1998). Amplification of 2 μl of the RT reaction was according to standard procedures, with 25 pmol of each primer in a final volume of 50 μl using 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and a final extension of 8 min at 72°C. Amplification products were examined by agarose gel electrophoresis. The PCR products were cloned directly into pGEM 3Zf(−) (Promega) which had been linearized with *Sma*I. Recombinant plasmids were purified using Qiaprep Spin minprep kits (Qiagen). Sequencing was performed on a Pharmacia ALF Express automated sequencer using Cy-5-labeled primers and a Thermosequenase cycle sequencing kit (Amersham). Each individual clone was sequenced until an unambiguous sequence was obtained so that clones derived from variant viruses could be identified. A consensus sequence for the viral population present in the serum at the time of sampling was obtained by direct sequencing of 100 ng of the purified, uncloned PCR product using Cy-5-labeled primers and a Thermosequenase cycle sequencing kit as before. DNA sequence data was analyzed using the GCG package and programs available on the SEQNET facility (Devereux *et al.,* 1984).

In situ hybridisation

BVDV-specific digoxigenin-labeled riboprobes were used to show the location of the viral RNA in the fixed tissues as previously described (Desport *et al.,* 1994). In brief, the sections were dewaxed and rehydrated to H_2O before treating with 100 μg ml^{-1} proteinase K at 37°C for 15 min to assist with the probe penetration. The sections were then hybridized in 50 μl of 50% formamide, 5% dextran sulfate, 2× SSC, 0.1 mM EDTA, 1 mM Tris–HCl, pH 7.5, 4 mg ml^{-1} denatured salmon sperm DNA, and 25 ng of riboprobe. The sections were coverslipped and initially denatured at 80°C for 10 min followed by hybridization for 2 h at 55°C using a Hybaid Omnislide block. Posthybridization washes were performed to remove any nonspecific binding and the probes were detected with polyclonal sheep anti-digoxigenin Fab fragments directly conjugated with alkaline phosphatase (Boehringer Mannheim). The enzyme activity was detected using the NBT/BCIP substrate and sections were left overnight for the blue signal to develop. The sections were counterstained with 1% neutral red, briefly cleared in acetone and ethanol, and mounted.

Virus neutralization assay

The presence of BVDV-specific virus neutralizing antibodies in the serum of the animals used in this study was determined by assessing the inhibition of growth of cytopathogenic BVDV strain NADL using a modified microtiter assay (Frey and Liess, 1971). Briefly, 100 $TCID_{50}$ of virus was added to doubling dilutions of antisera in culture media and incubated for 1 h at room temperature. Calf testis cells were then added and incubated for 5 days. Viral replication was detected using a swine anti-BVDV serum followed by a goat anti-swine serum coupled to horseradish peroxidase. The substrate TMB was used and the OD_{405} was read after stopping the reaction with 2 M H_2SO_4. The neutralizing antibody titer is the dilution of serum which gave a 50% inhibition of virus production. In some cases where neutralizing antibody was detected, a further assay was performed using homologous virus purified from the serum sample in place of the NADL strain.

Immunocytochemistry

Cryosections were cut into superfrost slides at 4–6 μm and allowed to air dry. The sections were fixed in acetone at 4°C for 10 min. The reagents were obtained from DAKO and used as directed. The sections were stained using an APAAP method with the following monoclonal antibodies: WB214 (specificity for E2, the major envelope glycoprotein, and used diluted in Tris-buffered saline (TBS) at 1:200), WB210 (specificity for the second envelope glycoprotein, E^{rns}, and used at 1:50), WB103 (specificity for the NS2/3 nonstructural protein, and used at 1:1000), and as a negative control antibody TRT 1 (specificity for an unrelated turkey rhinotracheitis virus protein and used at 1:20).

Briefly, sections were incubated with the primary antibodies at 37°C for 1 h. After washing in TBS, the sections were incubated with rabbit anti-mouse immunoglobulins followed by the mouse APAAP for a further 30 min each. After a final wash, the BCIP/NBT/INT substrate plus levamisole was allowed to develop the sections for 10 min. The slides were washed in water, counterstained in hematoxylin, and mounted in Faramount.

Oligonucleotides

The primer sequences used were obtained from BVDV consensus sequences from the EMBL and GenBank databases and, where available, known sequences of Pe515nc. The SP6 + SP3 pair specifically amplify the majority of the E2 sequence (lacking the transmembrane domain), which is known to contain antigenic domains. Oligo9 + oligo2 amplify the first 460 nucleotides of the NS3 region. The NS3 region is one of the most conserved between viral isolates and was chosen as a control for the experiment.

SP6, 5′ AGGGGCCAGATGGTACAGGGC 3′ (SD1 2405-2425);

SP3, 5′ GTCTACTAATCTGTAGCCAGTCTCATT 3′ (SD1 3175-3149);

oligo2, 5′ GACCATCCTTTCAAGTTTTTT 3′ (SD1 5620-5640); and
oligo9, 5′ GAGCACGAAAAAATGCCAC 3′ (SD1 5197-5180) were used.

ACKNOWLEDGMENTS

We are grateful to Dr. D. J. Paton (VLA, Weybridge) for the supply of the monoclonal antibodies WB103, WB210, and WB214 and for valuable discussion of this work. We also thank staff at the Institute for Animal Health at Compton for their contributions, particularly Mr. M. C. Clarke for the virus neutralization data, Mrs. B. V. Jones for the production of monoclonal antibody TRT1, Mrs. S. M. Hacker and Mrs H. I. Cook for their excellent assistance in the histological preparation of samples, and the compound staff (at IAH) for the care of the animals. The technical support provided by Ms. N. J. Archer throughout this project was invaluable.

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