Role of bovine viral diarrhea virus biotype in the establishment of fetal infections

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Objective—To examine the role of bovine viral diarrhea virus (BVDV) biotype on the establishment of fetal infection in cattle.

Animals—30 mixed-breed pregnant cows.

Procedure—Pregnant cows were inoculated oronasally with either i-VVNADL, originating from a infectious BVDV cDNA clone of the National Animal Disease Laboratory (NADL) isolate, or the parental virus stock, termed NADL-A.

Results—All cows developed neutralizing antibodies to BVDV, and virus was commonly isolated from peripheral blood mononuclear cells or nasal swab specimens of NADL-A inoculated cows; however, virus was rarely isolated from specimens of i-VVNADL inoculated cows. i-VVNADL did not cause fetal infection, whereas all fetuses harvested from NADL-A inoculated cows at 6 weeks after inoculation had evidence of infection. Immunoblot analysis of fetal virus isolates revealed the absence of NS3, confirming a noncytopathic (NCP) biotype BVDV in the NADL-A stock. The sequence of the NCP contaminant (termed NADL-1102) and the i-VVNADL genome were virtually identical, with the exception of a 270-nucleotide-long insert in the i-VVNADL genome. Phylogenetic analyses revealed that NADL-1102 forms a monophyletic group with 6 other NADL genomes.

Conclusions and Clinical Relevance—These data suggest that the contaminating NCP virus in the NADL stock was the ancestral NADL virus, which originally infected a bovine fetus and recombined to produce a cytopathic (CP) variant. Following oronasal inoculation of pregnant cows, viremia and transplacental transmission of CP BVDV to the fetus is rare, compared with the high occurrence of maternal viremia and fetal infection observed with NCP BVDV. (Am J Vet Res 2002;63:1455–1463)

Ruminant and swine pestiviruses are ubiquitous and an important cause of economic losses in most countries. Bovine viral diarrhea virus (BVDV) is a member of the Pestivirus genus of the Flaviviridae family and is associated with reproductive losses and enteric and respiratory disease in cattle. Bovine viral diarrhea virus is closely related to viruses that cause border disease in sheep and classic swine fever in pigs. Pestiviruses are divided into 2 categories or biotypes. Those of the cytopathic (CP) biotype induce cytopathologic changes in cultured cells, whereas viruses of the noncytopathic (NCP) biotype do not. Most BVDV circulating in cattle herds have a NCP phenotype.

Although embryonic and fetal death are known to follow BVDV infection in early gestation, most infected fetuses survive. Fetuses infected during the second trimester may have no anatomic damage or develop with anomalies of varying degrees, such as cerebellar, ocular, and skeletal degeneration, including cerebellar hypoplasia, hydranencephaly, cataracts, retinal degeneration, optic neuritis, and mandibular brachygnathism. Fetuses that survive BVDV infection during early gestation develop into calves that are born persistently infected (PI) and immunotolerant. These cattle shed virus continuously and serve as the most important source of virus spread. Many PI cattle succumb to mucosal disease when a mutation occurs in the infecting NCP virus that renders it cytopathogenic, creating an endogenous superinfection with CP BVDV. With rare exceptions, PI cattle with clinical mucosal disease constitute the only known natural source of CP BVDV, and these isolates are often contaminated with NCP BVDV. The biologically cloned NCP and CP BVDV components from such infected cattle are often known as NCP-CP BVDV pairs. In contrast, NCP virus can be isolated free of CP agents from PI cattle or infected fetuses. Bovine viral diarrhea virus is endemic in most of the world; in these areas 1 to 2% of the cattle can be PI. The need to understand BVDV fetal transmission has emerged as a result of efforts to eradicate the disease from endemic areas. A solid knowledge of the biologic characteristics of vertical transmission of BVDV will be necessary to develop

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vaccines that protect the fetus and to design effective eradication plans.

The purpose of the study presented here was to examine the role of BVDV biotype on the establishment of fetal infection. To this end, we studied fetal infection with either wild-type (ie, NADL-A) or infectious clone-derived BVDV (ie, i-VVNADL) in cows inoculated oronasally during the early second trimester of gestation. We report on the remarkable impact of BVDV biotype on the establishment of fetal infection.

Materials and Methods

Viruses and cells—The NADL-A reference strain was originally isolated from the spleen of an animal with mucosal disease.24,25 The NADL-A isolate was propagated in a bovine testicular continuous cell line (RD420);26 and this stock was termed NADL-A in 1993. The i-VVNADL seed was prepared from a sample of RNA transcribed in vitro from a pVVNADL DNA template. Ribonucleic acid transcripts were electroporated into RD420 cells to recover live BVDV, as described.27 Challenge viruses were propagated in Madin-Darby bovine kidney (MDBK) cells (clone BK-6); Viruses were titered by endpoint dilution in RD420 cells, as described.28 All tissue culture reagents purchased were from a single vendor, unless otherwise specified.29 BK-6 cells were propagated in an enhanced minimum essential medium that contained 0.01% bovine serum albumin and gentamicin sulfate (50 µg/mL). RD420 cells were grown in minimal essential medium that contained 1% antibiotic-antimycotic and 5% fetal equine serum.29 Bovine turbinate cells, grown in Dulbecco minimal essential medium (DMEM) supplemented with 5% fetal equine serum, were used for serum neutralization assays.30

Study design—Thirty mixed-breed BVDV seronegative pregnant cows (at 90 to 105 days of gestation) were used in our study. Fifteen cows were assigned to each of 2 isolation rooms and allowed to acclimate for 7 days. Ten cows were inoculated via intranasal aerosol of 10 mL of cell culture supernatant containing 107 median tissue culture infective doses (TCID50) of i-VVNADL (ie, infectious clone-derived BVDV) or NADL-A (ie, wild-type BVDV).31 By convention, we called this postinoculation day 0. Five cows in each group were mock-infected and remained as sentinels that were housed in the same room as infected cows. Health status of cows was evaluated daily, and rectal temperature was recorded. Venous blood was collected 2 times a week in EDTA-containing 3 times a week and were immediately placed in transport medium, consisting of 2 mL of DMEM plus 2% antibiotic-antimycotic (final concentrations: penicillin G sodium, 200 µg/mL; streptomycin sulfate, 200 µg/mL; amphotericin B, 500 ng/mL) and 5% fetal equine serum.32 A total of 15 fetuses were harvested from principal and control cows at either postinoculation day 21 or 42. The mock-inoculated cows and those inoculated with the NADL-A underwent cesarean surgery to remove the fetus. Cows inoculated with i-VVNADL, which had been prepared by reverse genetic approaches, were euthanatized to collect fetal and mock-inoculated cows and those inoculated with the NADL-A biotype, determined by NS3 expression, on hemotropism and establishment of fetal infection.

Serum neutralization and virus isolation assays—Neutralizing anti-BVDV antibody was titered in serum, using a serum neutralization assay, as previously described.33 The assay challenge virus consisted of 100 TCID50 of CP 5960 BVDV type 1 in 0.1 mL.

For virus isolation, peripheral blood mononuclear cells (PBMCs) were harvested from 4 mL of whole blood by use of a non-ionic density gradient,34 essentially as described previously.35 The PBMC layer in the gradient was harvested and washed in Hanks balanced salt solution and 10% sodium citrate. Approximately a third of the cell volume was used for cocultivation with monolayers of RD420 cells in 25 cm2 flasks. Cellular debris was removed from the nasal swab specimens by centrifuging at 850 × g for 15 minutes at 4°C; supernatant was used to inoculate monolayers of RD420 cells in 25 cm2 flasks. Tissues were weighed and a 1% homogenate was prepared by crashing in plastic bags. Approximately 3 mL of clarified supernatant was used to inoculate monolayers of RD420 cells in 25 cm2 flasks. Processed tissue specimens underwent 2 blind passages followed by inoculation of monolayers in 96-well plates for subsequent detection of BVDV by immunohistochemistry analysis. On the third day of incubation, monolayers were fixed and stained with a goat anti-BVDV specific antiserum, washed 4 times, and bound antibody was detected by use of a rabbit anti-goat peroxidase conjugate with an amino ethyl carbazole substrate.36

Immunohistochemistry analysis—Paraffin-embedded tissues were sectioned, deparaffinized in xylene, rehydrated in alcohols, and treated with protease in 0.3M tris-buffered saline solution for 15 minutes at 37°C. Sections were blocked for 30 minutes at room temperature (approx 18 to 24°C) in tris-buffered saline solution with 4% horse serum.37 Anti-BVDV monoclonal antibody (Mab) 15C5 was added at a 1:1,000 dilution and incubated for 1 hour at room temperature (approx 18 to 24°C).38 Slides were washed and biotinylated anti-mouse IgG was added. Slides were rinsed and incubated with horseradish peroxidase-conjugated avidin-biotin complex.39 Bound conjugate was detected after rinsing slides in saline (0.9% NaCl) solution, using peroxide as substrate and a diaminobenzidine-based indicator dye to develop a colored precipitate. Slides were counterstained in hematoxylin.

Isolate identification and determination of isolate cytopathogenicity—A NCP BVDV, which was isolated from the PBMCs collected postinoculation day 18 from a cow inoculated with NADL-A, was designated NCP-1102 and selected as a representative NCP virus for the molecular analyses. Following an additional passage of NCP-1102 in RD420 cells, indirect immunofluorescence staining was performed by use of Mab 15C5 (BVDV-specific) and 20.10.6 (pestivirus-specific).40 Staining of NCP-1102 was compared with the CP biotypes by use of a plaque assay and identification of the NS3 (p80) protein in western blots as described.41 The plaque assay was performed on RD420 monolayers. For western blotting, proteins were separated by SDS-PAGE, blotted onto nitrocellulose membranes, and blocked in phosphate-buffered saline (PBS) solution that was supplemented with 0.05% Tween 20 and 3% dry milk. All incubations were performed at room temperature (approx 18 to 24°C) for 1 hour. Three washes in PBS solution and 0.05% Tween 20 for 5 to 10 minutes each were performed between each incubation. The primary antibody was Mab 20.10.6, and the secondary antibody was a goat anti-mouse IgG tagged with horseradish peroxidase. Both were added at a 1:10,000 dilution in PBS solution (supplemented with Tween 20 and dry
milk), and bound conjugate was detected by use of a chemiluminescent substrate.

Nucleotide sequencing and data analysis—Ribonucleic acid was isolated from BVDV NADL-1102 infected cells by use of a guanidinium-phenol extraction method and reverse transcriptase polymerase chain reaction (RT-PCR) assay performed as described. The following oligonucleotides were used as primers, with nucleotide sequences derived from the Genbank NADL accession No. M31182 (nucleotide range as indicated, complementary strand denoted by rev): 2717 (2717 to 2746), 6322 (6322 to 6350/rev), 9307 (9307 to 9325), N55Brev (12333 to 12352/rev). These primers generated amplicons of 3.4 and 3.0 kilobases, respectively. Amplification protocols used exonuclease-containing thermostable polymerase mix for proof-reading and high fidelity. Sequencing was performed by use of amplicon DNA templates in chain termination reactions with fluorescent-labeled dideoxy nucleotides and analyzed in a sequencing instrument. Nucleotide sequence comparisons were performed by use of the basic local alignment search tool (BLAST) algorithm to identify the most closely related viral isolates in the Genbank database. The genetic distance matrix was calculated after alignment of the nucleotide sequences by use of a software program. Phylogenetic analyses of sequence data were performed by use of a software package that employs the maximum parsimony method to generate phylogenetic trees. The trees with the shortest lengths were found by implementing the branch-and-bound search option.

Results

BVDV replication at the site of inoculation and viremia in infected dams—The ability of the inoculated NADL-A and i-VVNADL to undergo local replication and cause viremia was evidenced by their isolation from nasal swab specimens and blood samples. From the i-VVNADL inoculated cows, 7 of 80 (9%) nasal specimens, collected up to postinoculation day 21, contained detectable virus (Table 1). These swab specimens were collected from only 4 cows. In contrast, 31 of 80 (39%) of the specimens, collected in the same period, from NADL-A inoculated cows yielded virus. All (100%) NADL-A inoculated cows yielded virus from the nasal swab specimens on postinoculation day 6. Similarly, 1 of 80 (approx 1%) of the blood samples from i-VVNADL inoculated dams contained virus, compared with 22 of 80 (28%) of those collected from NADL-A inoculated cows. On postinoculation day 8, 8 of 10 NADL-A inoculated cows were viremic. Virus was not detected in any specimens originating from cows in the i-VVNADL inoculated group after postinoculation day 11, whereas 4 cows in the NADL-A group harbored virus in the blood samples on postinoculation day 14. One sentinel control cow, belonging to the NADL-A infected group, became viremic by postinoculation day 15 (data not shown).

Humoral immune responses in dams inoculated with BVDV—All inoculated cows developed a humoral immune response to BVDV within 3 weeks after infection (Fig 1). The median neutralizing antibody titer at postinoculation day 21 was 50 in the i-VVNADL group and 63 in the NADL-A group. Some differences were observed in the kinetics of the antibody response, whereas none of the cows inoculated with i-VVNADL had antibodies to BVDV at postinoculation day 13, 3 of 10 cows inoculated with NADL-A had seroconverted by postinoculation day 13, with titers of 16. There was no apparent correlation between serum neutralizing antibody titer and the hematotropism of the virus. In contrast, sentinel cows remained seronegative, including the sole viremic cow.

Fetal BVDV infection following oronasal exposure of dams to NADL-A and i-VVNADL—To compare the fetal tropism of NADL-A and i-VVNADL, fetal tissues and membranes were harvested on postinoculation day 21 or 42. No gross pathologic changes were observed during the necropsy of the i-VVNADL inoculated dams, and virus was not recovered from maternal tissue specimens (Table 2). NADL-A inoculated cows were not euthanatized, rather fetuses were retrieved by caesarian, except for 1 fetus that had a dam that died from a large lung abscess. No lesions were observed in any fetus from either the i-VVNADL or NADL-A inoculated cows. With the exception of a single instance of virus recovery from a placental specimen, no virus was isolated from any fetal tissues obtained from the dams.
inoculated with i-VVNADL. In contrast, BVDV was recovered from 8 of 10 fetuses and placentas harvested from NADL-A inoculated dams. Essentially, identical results were obtained by immunohistochemistry analysis of tissue specimens to identify BVDV antigen with a Mab that binds the E<sup>rm</sup> envelope glycoprotein. The agreement between the 2 approaches to establish viral infection was > 89%, although virus isolation proved slightly more sensitive than immunohistochemistry analysis. Rates of fetal infection were correlated with the frequency of local and systemic infection in each of the groups. The rare isolation of BVDV from maternal swab specimens and blood samples from dams inoculated with i-VVNADL correlated with the absence of fetal infection. Conversely, NADL-A inoculated cows supported abundant virus replication at these sites, which correlated with the finding that all fetuses were infected.

All virus isolates from maternal specimens collected antemortem, as well as from maternal and fetal specimens harvested postmortem from the i-VVNADL inoculated cows, induced extensive in vitro cytopathologic changes, which were consistent with the CP nature of a virus that was derived by reverse genetics from a cloned cDNA. Noncytopathic virus could not be detected in these specimens by use of BVDV immunohistochemistry analysis. Surprisingly, cell cultures inoculated with fluids from clinical or necropsy specimens derived from cattle inoculated with i-VVNADL induced extensive in vitro cytopathologic changes, which were consistent with the CP nature of a virus that was derived by reverse genetics from a cloned cDNA. Noncytopathic virus could not be detected in these specimens by use of BVDV immunohistochemistry analysis. Surprisingly, cell cultures inoculated with fluids from clinical or necropsy specimens derived from cattle inoculated with NADL-A revealed CP, NCP, or both biotypes; cells without evidence of CP BVDV had viral antigen detected by immunohistochemistry analysis. In fact, only nasal swab specimens collected on postinoculation day 8 or earlier from NADL-A inoculated cows yielded CP BVDV. The postinoculation day-8 specimens harbored NCP and CP virus. Only NCP BVDV was isolated from specimens harvested after postinoculation day 8. Cytopathic BVDV was never recovered from the blood samples and fetal tissue specimens from NADL-A inoculated cows.

To confirm the presence of a mixture of CP and NCP BVDV in nasal swab specimens, a plaque assay was performed and developed by use of immunohistochemistry analysis of the cell monolayer with antibodies to BVDV and peroxidase-conjugated anti-IgG, followed by development with a chromogenic substrate. Wells were inoculated with the log 10 dilutions indicated. Results indicate the presence of mixed cytopathic (CP) and noncytopathic (NCP) BVDV infection. Notice in the -4 dilution well (white arrow) a focus of NCP BVDV infection in the monolayer that lacks the clear center typical of CP BVDV.

### Table 2

<table>
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<th>Tissue specimens</th>
<th>i-VVNADL</th>
<th>i-VVNADL&lt;sup&gt;†&lt;/sup&gt;</th>
<th>NADL-A</th>
<th>NADL-A&lt;sup&gt;†&lt;/sup&gt;</th>
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<td>VI (3 wk)</td>
<td>IHC (6 wk)</td>
<td>VI (6 wk)</td>
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<tr>
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<td>1 CP</td>
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<td>0</td>
</tr>
<tr>
<td>Maternal lung</td>
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<td>0</td>
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<td>0</td>
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</tr>
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<td>Fetal eye</td>
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<sup>†</sup>Number of animals yielding BVDV by virus isolation or by immunohistochemistry analysis in a group of 5. 1Number of animals in this group was 4.

IHC = Immunohistochemistry analysis. VI = Virus isolation. NT = Not tested.

See Table 1 for remainder of key.

Figure 2—Plaque assay results for a nasal swab specimen obtained on postinoculation day 8 from a cow inoculated with NADL-A. A plaque assay was performed and stained with antibodies to BVDV and peroxidase-conjugated anti-IgG, followed by development with a chromogenic substrate. Wells were inoculated with the log 10 dilutions indicated. Results indicate the presence of mixed cytopathic (CP) and noncytopathic (NCP) BVDV infection. Notice in the -4 dilution well (white arrow) a focus of NCP BVDV infection in the monolayer that lacks the clear center typical of CP BVDV.
Molecular and cellular analysis of viral isolates—
To further characterize the NCP virus recovered from NADL-A inoculated cows, isolates from viral culture of PBMCs and nasal swab specimens were inoculated onto cultured bovine cells to analyze the expression of NS3. NS3 is expressed efficiently only in cells infected with CP BVDV (Fig 3). Immunoblot analysis by use of a NS3-specific Mab revealed a vast excess of NS2–3 (p125), compared with NS3 (p80), in representative specimens collected on or after postinoculation day 11 (lanes 3 to 9), and originated from peripheral blood mononuclear cells (PBMCs; lanes 3, 7, and 8) or nasal swab specimens (lanes 4, 5, 6, and 9). Lanes 3 to 9 represent analysis of specimens from the following cows: lane 3 and 4, cow 1101; lane 5, cow 1107; lane 6, cow 1110; lane 7, cow 1101; lane 8 and 9, cow 1105. Panel B) All analyzed specimens were NCP in tissue culture, except for a CP control in lane 1 (stock i-VVNADL). Cells were inoculated with specimens collected at postinoculation day 15 (lanes 2 to 5), 18 (6,7) or 20 (8), and originated from PBMCs (2, 4, 5, 6, and 8) or nasal swab specimens (3 and 7). Lanes 2 to 8 represent analysis of specimens from the following cows: lane 2 and 3, cow 1101; lane 4, cow 1103; lane 5, cow 1105; lanes 6, 7, and 8, cow 1102 (infected sentinel).

Figure 3—Expression of NS3 by representative BVDV isolates from dams inoculated with NADL-A. Bovine cells were infected with the isolates as indicated, and lysates were analyzed by immunoblotting with a monoclonal antibody (Mab) that binds to the NS3 region of the polyprotein. Panel A) All analyzed specimens were NCP in tissue culture, except for CP controls in lane 1 (stock i-VVNADL) and lane 2 (nasal swab specimen from i-VVNADL inoculated cow 1091). Cells were inoculated with specimens collected at postinoculation day 11 (lanes 3 to 6) or 13 (7 to 9), and originated from peripheral blood mononuclear cells (PBMCs; lanes 3, 7, and 8) or nasal swab specimens (lanes 4, 5, 6, and 9). Lanes 3 to 9 represent analysis of specimens from the following cows: lane 3 and 4, cow 1101; lane 5, cow 1107; lane 6, cow 1110; lane 7, cow 1101; lane 8 and 9, cow 1105. Panel B) All analyzed specimens were NCP in tissue culture, except for a CP control in lane 1 (stock i-VVNADL). Cells were inoculated with specimens collected at postinoculation day 15 (lanes 2 to 5), 18 (6,7) or 20 (8), and originated from PBMCs (2, 4, 5, 6, and 8) or nasal swab specimens (3 and 7). Lanes 2 to 8 represent analysis of specimens from the following cows: lane 2 and 3, cow 1101; lane 4, cow 1103; lane 5, cow 1105; lanes 6, 7, and 8, cow 1102 (infected sentinel).

Figure 4—Expression of NS3 by representative isolated of fetal specimens from dams that were inoculated with NADL-A. Bovine cells were infected with the isolates as indicated, and lysates were analyzed by immunoblotting with a Mab that binds to the NS3 region of the polyprotein. All analyzed specimens were NCP in tissue culture, except for CP controls in lane 1 of panels A and B (stock i-VVNADL). Panel A) Immunoblot of cells infected with fetal BVDV isolates (cow 1101) that were harvested on postinoculation day 21 (lanes 3 to 6) or 23 (7 to 9), and originated from peripheral blood mononuclear cells (PBMCs; lanes 3, 7, and 8) or nasal swab specimens (lanes 4, 5, 6, and 9). Lanes 3 to 9 represent analysis of specimens from the following cows: lane 3 and 4, cow 1101; lane 5, cow 1107; lane 6, cow 1110; lane 7, cow 1101; lane 8 and 9, cow 1105. Panel B) All analyzed specimens were NCP in tissue culture, except for a CP control in lane 1 (stock i-VVNADL). Cells were inoculated with specimens collected at postinoculation day 15 (lanes 2 to 5), 18 (6,7) or 20 (8), and originated from PBMCs (2, 4, 5, 6, and 8) or nasal swab specimens (3 and 7). Lanes 2 to 8 represent analysis of specimens from the following cows: lane 2 and 3, cow 1101; lane 4, cow 1103; lane 5, cow 1105; lanes 6, 7, and 8, cow 1102 (infected sentinel).

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To obtain molecular information to help establish the source of the NCP BVDV, we analyzed the nucleotide sequence of the viral genomic RNA. Viral RNA from the cell cultures infected with an isolate from a NADL-A infected cow was amplified by use of a RT-PCR assay and the nucleotide sequence of the NS2–3 and NS5A regions determined. Size and sequence analysis of an amplicon DNA of 1 kilobase and including position 4993 (C-terminus of NS2) indicated that the NADL-1102 lacked the 270 base pair bovine cellular insert element near the NS2 C-terminus (Fig 4). Nucleotide sequence comparisons indicated a > 99.9%
similarity between NCP-1102 and the NADL prototype sequence in the 500 base pair segment flanking the NADL 270 base pair insert. To establish the relationships of NCP-1102 to other BVDV, its sequence was compared with all the sequences in the Genbank database. A phylogenetic analysis of NADL-1102 and 9 additional pestivirus sequences performed by the maximum parsimony method indicated that the NCP-NADL-1102 relationship was translationally silent. A phylogenetic analysis of NADL-1102 and 9 additional pestivirus sequences performed by the maximum parsimony method indicated that the NCP-NADL-1102 relationship was translationally silent.

Figure 5—Phylogram of the consensus evolutionary tree of BVDV genomes most closely related to NADL-1102. Sequences from nucleotide 10190 to 12334 (2144 nucleotides encoding NS5B) were analyzed by use of software, which uses a maximum parsimony algorithm. The tree branches are labeled with the Genbank accession numbers of the pestivirus isolates as in Table 3. Border disease virus, strain 31 was added to the alignment as an outgroup to root the tree (Genbank accession U70263). The total length of the shortest tree was 838 steps. The consistency index (proportion of changes caused by forward mutations) is 0.971. The horizontal distance is proportional to the minimum number of nucleotide differences needed to join nodes and BVDV sequences.

BVDV from cattle with mucosal disease. Lower nucleotide sequence identity values are typical of comparisons made to independent isolates, such as CP Singer BVDV in this case. Analysis of the nucleotide substitutions that separate the CP NADL prototype sequence (Genbank accession No. M31182) and the NADL-1102 in a region of the genome encoding NS5B-B revealed that most of them consist of transition mutations (Table 4). Moreover, most substitutions were either translationally silent or resulted in conservative amino acid changes. The only nucleotide substitution found in the 1-kilobase region of the genome encoding part of NS2–3 and encompassing the bovine segment insertion was translationally silent.

Table 3—Nucleotide sequence identity* between NADL-1102 and the most similar genomes in Genbank

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*Values indicate percentage identity to the NADL-1102 isolate in a 2144 nucleotide segment (ie, nucleotides 10190 to 12334).

Table 4—Impact of sequence differences between NADL prototype and BVDV isolate 1102

<table>
<thead>
<tr>
<th>Region sequenced</th>
<th>Nucleotide(s) in NADL*</th>
<th>Nucleotide in NADL-1102</th>
<th>Translation‡</th>
<th>Type of substitution</th>
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<td>NS2-3 (4694-5264)</td>
<td>4912-CCG CCA†</td>
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<td>NS5A (9971-10191)</td>
<td>10009-GAT GAC</td>
<td>Silent Transition</td>
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<td>NS5B (10192-11649)</td>
<td>10246-TTT TTC</td>
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<td>10411-CCT GTA</td>
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<td>10506-AGG AAG</td>
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<td>10546-CCA CCC</td>
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<td>10797-ACC GCC</td>
<td>T &gt; A Transition</td>
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<td>10838-GTA GTA</td>
<td>V &gt; I Transition</td>
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<td>V &gt; D Transition</td>
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<td>11196-TGG GGC</td>
<td>G &gt; A Transition</td>
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*Bases in boldface indicate mutation.
†Consequence of codon change in the translated protein product, with the NADL amino acid (in the single letter code of the International Union of Pure and Applied Chemistry) preceding the > symbol. $270 nucleotide sequence element (bovine origin). ‡NS2 expression absent in NADL-1102.
NADL sequences were available from a monophyletic group with the NADL-1102 sequence. The nucleotide sequence differences among virus stocks that underwent several cell culture passages in various laboratories over a period of several years are attributable to the natural variation that results from the high error rate of the viral RNA replicase. The NADL isolate originated from a calf with mucosal disease, and no systematic attempts to eliminate NCP BVDV were made by the repository personnel or the depositors.

Discussion

The broad implication of this work is that NCP NADL-A infects the bovine fetus following oronasal exposure of the dam, whereas the closely related CP NADL-A pair and i-VVNADL were incapable of fetal infection. The studies were performed by a natural route of virus exposure, oronasal inoculation of the dam, during the early stage of the second trimester of gestation. Further studies are needed to determine whether these observations are restricted to this particular stage of gestation and route of inoculation. The failure of CP BVDV to establish fetal infection following inoculation of pregnant cows is interesting, given the controversy in the literature. Several earlier published reports described fetal infections following inoculation of ruminants with various strains of CP BVDV. For example, Kendrick infected pregnant cows in the first trimester with NADL via IM injection and concluded, on the basis of fetal pathologic findings, that CP NADL was transmitted to the fetus. Results of another study indicated that direct inoculation of the uterus by injection of CP BVDV strain NADL across the uterine wall led to fetal infection and pathologic changes. Following direct inoculation of fetal membranes of 4 seropositive cows with CP BVDV (isolate Pe51Sc Cl), results of a prior study document that CP BVDV is a probable cause of stillbirths, without establishing persistent infection. Similar conclusions were obtained by oronasal inoculation into 5 heifers.

The interpretation of these results is complicated by the absence of a NCP BVDV infection as a control to establish the absence of cellular immunity in the dams. Alternatively, other sites of virus replication in i-VVNADL cows not examined in our study may generate sufficient viral antigen load in draining lymphoid tissues and stimulation of a more vigorous immune response.

In our study, NCP NADL-A was detected in PBMCs and nasal swab specimens up to postinoculation day 21, with the NADL reference stock thought to contain CP BVDV exclusively. In contrast, CP NADL, inoculated as either i-VVNADL or the NADL-A stock, was rapidly cleared from the site of inoculation and the blood samples by postinoculation day 8, suggesting that the CP BVDV infectious dose that reached the fetus may have been considerably lower than the NCP counterpart. Alternatively, protection to the fetus may be provided by inhibition of CP BVDV replication by nonspecific mechanisms such as the production of interferon-α or -β. The latter seems more likely, in the light of a recent report indicating that type-I interferon is abundant in the amniotic fluid of fetuses inoculated with CP BVDV. Interestingly, interferon is scarce in the fetus infected with NCP BVDV, suggesting a role for fetal interferon in preventing the establishment of fetal BVDV infection. It should be noted that whereas 3 of 5 NADL-A inoculated cows had infected fetuses by postinoculation day 21 (day 121 of gestation), the proportion rose to 5 of 5 cows by postinoculation day 42 (day 142 of gestation). This suggests a rather delayed establishment of fetal infection following maternal inoculation. This finding is at odds with results of experimental studies performed in sheep. These differences may be ascribed to the impact of animal species, viral isolates, and experimental protocols in the outcome of fetal infection.

There are several contrasting elements of virus-host interactions that exist in CP and NCP infections in cattle. The CP isolates are rapidly cleared from maternal circulation (ie, postinoculation day 8 as compared with postinoculation day 21 for NCP BVDV) and may even be rapidly cleared from fetal tissues, thus never establishing a productive infection. The mechanisms for the rapid in vivo resolution of CP BVDV infections, compared with the NCP biotype, begin to be elucidated in vitro. It has been documented that amount of interferon-α or -β expression induced by double stranded RNA are reduced following in vitro infection with NCP BVDV. The CP BVDV induces host cell apoptosis in vitro, rather than killing cells by direct lytic events. Apoptosis mediated by CP BVDV infection correlates with increased RNA accumulation. These features are associated with NS3 expression. It is possible that the accumulation of apoptotic cells in vivo following CP BVDV infection provides alarm signals (eg, interferon induction, as well as an abundant source of viral antigen for uptake by dendritic cells) and subsequent CD8+ T cell activation by cross-priming. Despite limited replication in the host, i-VVNADL elicited humoral responses comparable to those of NADL (a mixture of CP and NCP), suggesting that antigen presentation by CP BVDV is efficient. Post-apoptotic events may promote CD4+ Th2 cell activation and drive antibody responses, accounting for the comparable antibody concentrations attained in NADL-A and i-VVNADL inoculated cattle. Alternatively, other sites of virus replication in i-VVNADL cows not examined in our study may generate sufficient viral antigen load in draining lymphoid tissues and stimulation of a more vigorous immune response.

These studies led us to conclude that the NCP virus identified in fetuses is the NCP precursor of the CP NADL isolate. Thus, the stock of the NADL strain of BVDV used to inoculate cows, thought to contain
only CP virus, most likely contained an unsuspected NCP BVDV. It is technically difficult to confirm the absence of a relatively small proportion of NCP BVDV in stocks of CP BVDV by use of in vitro laboratory methods, especially when the molecular differences between the 2 genomes are not known. In our study, the phenomena of in vivo maternal and fetal tissue restriction of CP BVDV allowed the discovery of a NCP contaminant in the NADL-A reference stock.

Nucleotide sequence analyses revealed that the NADL-1102 contaminant is virtually identical to the prototype CP BVDV strain NADL, except for the absence of the 270 nucleotide bovine genome element.21 Nucleotide sequence conservation is greater in the NS2-3 region than in the NS5A-B region, which had 0.2 and 0.5% divergence, respectively (Table 3). This level of difference between 2 members of a pair of CP and NCP BVDV is attributable to quasi-species variation. Database searches by use of BLAST indicate that BVDV isolate 1102 is most closely related to NADL than to any other known BVDV isolate, and this level of nucleotide sequence identity is typical of CP-NCP BVDV pairs, as reported.13 Rearrangements that lead to host sequence insertion into the NS2 region of genome of NCP BVDV and acquisition of a CP phenotype is well documented.17 However, the converse has not been reported. Because reversion from CP to NCP is unprecedented, we hypothesize that the original NADL stock obtained from an animal with mucosal disease contained both BVDV biotypes. This theory is supported by published reports concluding that most tissue specimens from animals with mucosal disease tissue contain CP and NCP BVDV biotypes.14 Therefore, it is highly probable that the NCP-1102 isolated in our study is the NCP parent of NADL, and that a recent ancestor of NCP-1102 caused a fetal infection in an animal that later became PI. Eventually, the PI animal succumbed to CP BVDV-induced mucosal disease,22 and the CP BVDV isolated served as the seed for the NADL reference strain.14 These findings highlight the need to interpret previous research with uncloned stocks of NADL and other CP BVDV isolates with caution because of the possible presence of 2 viral biotypes. To avoid future confusion, we propose the use of NADLcp to refer to biologically cloned CP virus and NADLncp for the NCP pair. Obviously, the uncertainty surrounding the purity of viral stocks derived by biological cloning is circumvented with the use of BVDV recovered from cDNA cloned in plasmids.

The inability of CP BVDV to establish fetal infection following oronasal exposure explains the preponderance of NCP BVDV in the cattle population. The extraordinary ability of NCP BVDV to establish fetal infections leading to the birth of PI calves provides a robust viral reservoir. Shedding and horizontal transmission maintains a high degree of viral infection in cattle herds.21,25 Conversely, the paucity of CP BVDV isolation from PI cattle free of mucosal disease is the consequence of the failure of CP isolates to be transmitted vertically. This singular aspect of the biologic characteristics of CP BVDV suggests that an attenuated CP biotype BVDV used as a modified-live vaccine may reduce the risk of fetal infection in pregnant cattle.

References


16. Bolin SR, McClurkin AW, Cuthlip RC, et al. Severe clinical disease induced in cattle persistently infected with noncytopathic...


