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Monoclonal Antibody Analyses of Cytopathic and Noncytopathic Viruses from Fatal Bovine Viral Diarrhea Virus Infections

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A panel of monoclonal antibodies that recognize the two major glycoproteins of bovine viral diarrhea virus (BDV) was used to evaluate the antigenic relationship between cytopathic (CP) and noncytopathic (NCP) viruses isolated from cattle dead or dying from fatal BDV infections. Various unrelated BDV isolates were initially screened by indirect immunofluorescence with monoclonal antibodies directed against the 56- to 58- and 48-kilodalton glycoproteins of the virus. A wide spectrum of reactivity that was independent of biotype was found. Biological clones of the same isolate showed only minor variations from the parental isolate, as did isolates taken from different animals located on the same farm. A similar analysis was repeated with pairs of CP and NCP viruses isolated from 16 unrelated clinical cases of BDV infection resulting in fatal disease. The reactivity patterns within individual pairs of isolates taken from the same animals were in most instances very similar and in some cases indistinguishable from one another. The results demonstrate that antigenic similarity between biotypes is a consistent finding in animals dying from fatal BDV infections. In view of the wide degree of variation in reactivity patterns between unrelated BDV isolates, the close antigenic similarity of CP BDV to the homologous NCP BDV of a given pair strongly suggests that CP BDV arises by mutation from NCP BDV.

Bovine viral diarrhea virus (BDV), a positive-stranded and enveloped RNA virus, is currently considered to be a member of the genus *Pestivirus* in the family *Togaviridae*. Its genomic structure and replication strategy, however, suggest that it may in the future be classified as a member of the genus *Flavivirus* in the family *Flaviviridae* (13). The virus has a worldwide distribution and produces a broad range of clinical signs in cattle, depending on the age and immune status of the animal at the time of infection.

Both cytopathic (CP) and noncytopathic (NCP) viruses are routinely identified by cell culture techniques, but their combined role in the pathogenesis of fatal disease has only recently been elucidated. In most well-defined instances, both CP BDV and NCP BDV can be isolated from animals that have died with the classic lesions of bovine virus diarrhea. The mechanism by which these two biotypes of the virus induce fatal disease is unique among animal viruses and begins with an early transplacental infection of the fetus with NCP BDV prior to the complete maturation of the fetal immune system at about 120 days of gestation. Animals that survive fetal infection are frequently identified as persistently viremic, with no neutralizing antibody response (12). If these persistently infected animals are subsequently superinfected with a particular CP BDV, they develop fatal disease and exhibit the classic mucosal lesions of bovine virus diarrhea (2, 3; W. V. Corapi, R. O. Donis, and E. J. Dubovi, unpublished observation).

Even with this information, the ability to reproduce fatal disease experimentally in persistently infected cattle has been only sporadic at best (unpublished observation), and the exact relationship between pairs of CP BDV and NCP BDV isolates capable of inducing fatal disease has not been firmly established. In addition to the biotypic differences that exist, the virus also exhibits a great degree of antigenic variation, and there has been some speculation as to the importance of antigenic similarity between the two biotypes in the induction of fatal disease (10). That antigenic differences are important is evidenced by the fact that persistently infected animals are capable of responding immunologically to certain CP BDV isolates and not to others (1; unpublished observation). CP BDV and NCP BDV isolated from animals with fatal disease have also been shown to be antigenically indistinguishable by neutralization endpoint assays with polyclonal antiserum, while viruses from unrelated sources are usually antigenically different (10).

The two major glycosylated polypeptides present in BDV-infected cells have molecular sizes of 56 to 58 and 48 kilodaltons (kDa) (5). We have previously shown that neutralizing antibodies are directed against the 56- to 58-kDa protein, making it a likely candidate responsible for the antigenic variation that exists among various BDV isolates (4). Using a panel of monoclonal antibodies (MAbs) that recognize these two proteins, we screened a large number of unrelated BDV isolates in an indirect immunofluorescence assay (IFA) to determine the nature and extent of the antigenic variation that exists between isolates of either biotype. Numerous biological clones of the same virus as well as various viral isolates from the same farm were also examined.

Sixteen pairs of CP BDV and NCP BDV isolates from animals dead or dying from natural or experimental BDV infections were then examined for reactivity with the panel of MAbs. Antigenic similarity was seen in all pairs from natural infections but was less evident in pairs from experimentally induced or vaccine-induced cases. The results demonstrate that a high degree of antigenic similarity between both biotypes is a consistent finding in natural cases of fatal BDV infections and suggest a likely source for the origin of the CP biotype.

MATERIALS AND METHODS

MAb production. The Singer type strain of BDV was used to immunize mice for the production of lymphocytes to be fused with murine myeloma cells as previously described (4,

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8, 11). Positive hybridoma colonies were cloned twice by limiting dilution. Ascites fluid was produced by intraperitoneal injection of approximately 10^7 cells from each positive hybridoma clone into mice 24 h after intraperitoneal injection of 0.5 ml of Freund incomplete adjuvant to enhance tumor formation. All tests were carried out with ascites fluid collected from these mice.

Cell cultures and viruses. Hybridoma cells were cultured in RPMI 1640 medium and Dulbecco modified Eagle medium supplemented with 20% fetal calf serum, 50 μM 2-mercaptoethanol, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 10^{-4} M hypoxanthine, and 1.6×10^{-5} M thymidine. BDV type strains Singer, Oregon-C24V, and NADL were obtained from National Veterinary Service Laboratories, Ames, Iowa. NY-1 was obtained from the American Type Culture Collection, Rockville, Md. All remaining BDV isolates were taken from blood or tissue samples submitted to the Diagnostic Laboratory at the New York State College of Veterinary Medicine. All CP BDV isolates were cloned twice by plaque purification, while NCP BDV isolates were cloned twice by limiting dilution. All viruses were grown in bovine testicle cells cultured in Eagle minimum essential medium supplemented with 10% fetal bovine serum, 200 IU of penicillin per ml, 200 g of streptomycin per ml, and 2.5 g of amphotericin B (Fungizone; E. R. Squibb & Sons, Princeton, N.J.) per ml. All cell cultures and fetal calf serum were determined to be free of NCP BDV and mycoplasmas.

Immunofluorescence procedures. Positive hybridoma colonies were screened and cloned based on their reactivity in IFA with bovine testicle cells infected with the Singer type strain of BDV. The reactivity patterns of all BDV isolates with the panel of positive MAb were determined by IFA with bovine testicle cells infected with the different BDV isolates. Standardization of MAb reactivity between clones was accomplished by using the highest dilution of MAb that showed no significant loss in IFA staining against the Singer type strain of BDV. A BDV-specific fluorescein isothiocyanate-conjugated polyclonal antiserum (National Veterinary Service Laboratories) was used as a positive control for all isolates tested.

Virus neutralization. MAbs were used in a standard virus neutralization assay against 100 50% tissue culture infective doses of the Singer type strain of BDV, with bovine testicle cells as the indicator cells. Neutralization titers reported are the reciprocal of the highest dilution of MAb that inhibited cytopathology.

Radioimmunoprecipitation of infected cell extracts. BDV-infected cells were radiolabeled with [^{35}S]methionine. Virus-specific proteins were immunoprecipitated from the cytoplasmic cell extracts with the MAbs as previously described (4). All MAbs immunoprecipitated either the 56- to 58-kDa or 48-kDa glycoprotein of the virus (4).

RESULTS

Reactivity of MAbs with unrelated BDV isolates. A total of 60 different CP and NCP cloned BDV isolates were examined for their reactivity with a panel of 22 MAbs to ascertain the pattern and extent of antigenic variation existing among various isolates. The isolates examined were randomly selected from field cases in New York State and represent a wide range of clinical manifestations of BDV infections. Virus infected bovine testicle cells were tested by IFA with the panel of MAbs. The MAbs were grouped into 10 different classes recognizing 10 different virus-specific antigenic sites based on their pattern of reactivity with the viral isolates, neutralizing activity, and protein specificity (Tables 1 and 2). Radioimmunoprecipitation data documenting the protein specificity of the MAbs for the two major glycoproteins of the virus have been previously reported (4). Neutralization titers were determined as described in Materials and Methods. When more than one MAb is listed as recognizing a single antigenic site, the neutralization titer reported represents the mean value for all members of that class. Neutralization-resistant viral mutants were used to further confirm the unique specificity of each of the six neutralizing classes of MAb (data not shown).

A wide spectrum of reactivity ranging from very strong reactivity with all of the MAbs to weak reactivity with only a single MAb was found among the isolates. A semiquantitative evaluation of the degree of reactivity of each of the isolates with the panel of MAbs was made based on the intensity of fluorescence and the number of positive cells detected. Three different strains of BDV and their patterns

TABLE 1. Classification of MAbs specific for BDV

MAb	Antigenic site	Neutralization (titer)	Protein specificity (kDa)
1	1	−	48
2-5	2	+ (96,000)	56–58
6-9	3	+ (480,000)	56–58
10-13	4	−	56–58
14-17	5	−	56–58
18	6	+ (3,200)	56–58
19	7	+ (16,000)	56–58
20	8	−	56–58
21	9	+ (19,200)	56–58
22	10	+ (192,000)	56–58

TABLE 2. Reactivity patterns of selected BDV isolates with the panel of MAbs[a]

	Antigenic sites										
Isolates	PC	1	2	3	4	5	6	7	8	9	10
Singer CP	■	■	■	■	■	■	■	■	■	■	■
NADL CP	■	▒	■	■	■	−	▒	■	■	−	−
Oregon CP	■	■	▒	■	▒	▒	■	−	−	−	−
Isol 1 CP	■	■	−	−	■	▒	■	■	−	−	−
Isol 2 NCP	▒	▒	■	−	■	▒	■	■	−	−	−
Isol 3 CP	■	▒	−	■	▒	▒	▒	−	−	−	−
Isol 4 NCP	▒	▒	■	■	▒	▒	−	−	−	−	−
Isol 5 CP	▒	▒	■	■	−	▒	−	−	−	−	−
Isol 6 NCP	▒	▒	■	■	▒	−	−	−	−	−	−
NY-1 NCP	▒	▒	■	■	−	−	−	−	−	−	−
Isol 7 NCP	■	▒	▒	−	−	−	−	−	−	−	−
Isol 8 CP	▒	■	−	−	−	−	−	−	−	−	−

[a] Reactivity in IFA: −, negative; ▒, +1; ■, +2. Isol, Isolate. PC, BDV-specific polyclonal antiserum.

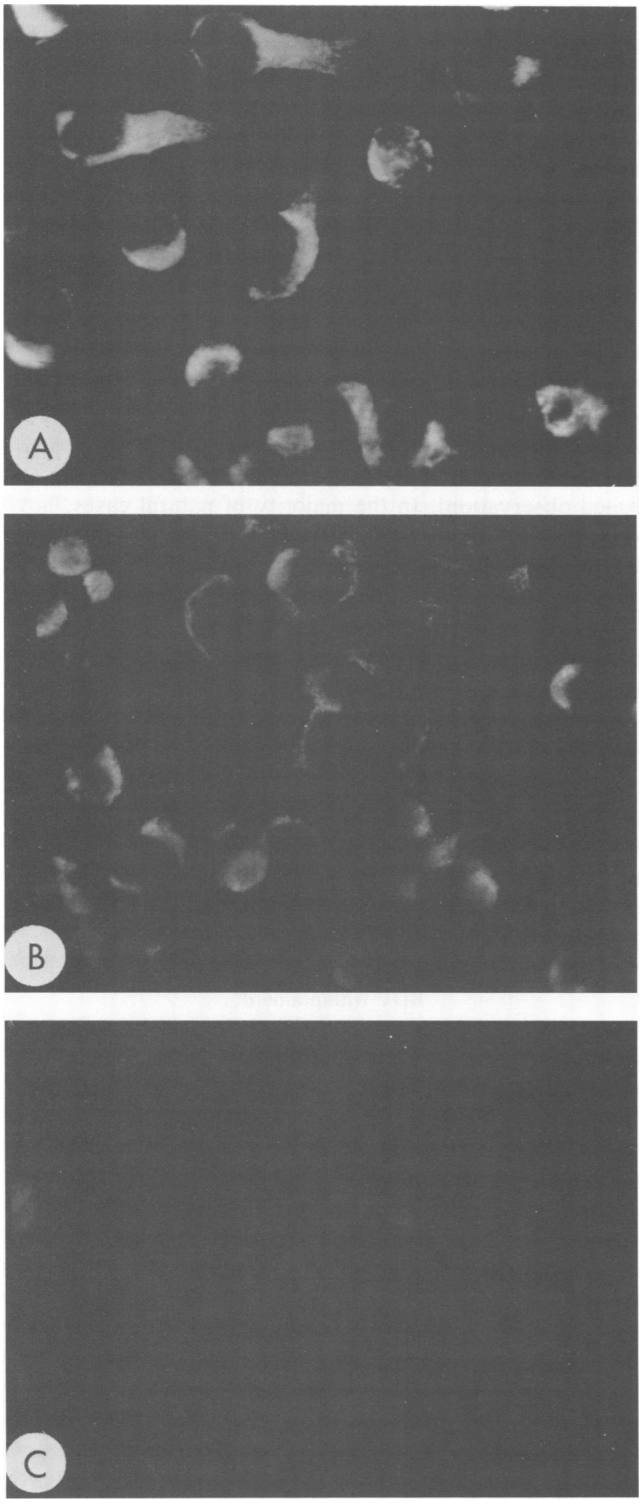


FIG. 1. IFA of BDV-infected cells. Bovine testicle cells were infected with the Singer type strain of BDV (A), the Oregon-C24V type strain of BDV (B), or a cytopathic field isolate of BDV (C). All cells were incubated for 16 to 24 h postinfection, fixed in acetone, and reacted with a single MAb specific for antigenic site 2 followed by fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin. The degree of reactivity was graded as +2 (A), +1 (B), and negative (C).

of fluorescence with a single MAb are shown to illustrate the degree of variation that exists (Fig. 1). The immunoreactivity patterns of eight field isolates and four type strains are shown in Table 2 as representative samples to illustrate the wide range of antigenic diversity that exists among different BDV isolates. Reactivity with a BDV-specific polyclonal antiserum (PC) is also shown in Table 2 as a positive control for all isolates.

Lack of antigenic variation among individual clones of the same isolate. Twenty separate biological clones derived from a single NCP BDV isolate were examined to determine whether antigenic variants are present in significant numbers within any single BDV isolate. The reactivity patterns of the various clones were indistinguishable from one another for all but 3 of the 20 clones (Table 3). Two of the clones which varied from the parental isolate were indistinguishable from one another and had a change in only one antigenic site (site 4), as evidenced by the loss of reactivity with the corresponding class of MAb. A single MAb reacted weakly with one additional antigenic site (site 8) in the remaining clone, which was not otherwise significantly different from the uncloned virus.

Antigenic relationship between CP and NCP pairs. Although antigenic similarities between CP BDV and NCP BDV pairs have been demonstrated serologically with neutralizing polyclonal antibodies (10), there are no reports of an MAb analysis of such pairs. CP BDV and NCP BDV pairs isolated from 16 different animals either dead or dying from BDV infections and exhibiting the typical clinical signs and mucosal lesions of bovine virus diarrhea were examined with the panel of MAbs (Table 4). The isolates recovered were submitted from a wide geographic area over a 3-year period from June 1984 to September 1987 and represented 14 natural cases (cases 1 to 14), one experimentally induced case (case 15), and one vaccine-induced case (case 16). In all 14 natural cases, the vast majority of the antigenic sites were conserved between the two biotypes of individual pairs. In 36% of these cases (cases 1, 3, 4, 9, and 13), the CP BDV and NCP BDV within a pair were indistinguishable from one another. The remaining cases had differences at one to three antigenic sites only. Cases 15 and 16, the two artificially induced cases, were the most dissimilar of the pairs examined and had differences at 3 and 4 of the 10 antigenic sites, respectively. The CP BDV isolates tended to react more strongly with the MAbs than did the NCP BDV isolates in all pairs examined.

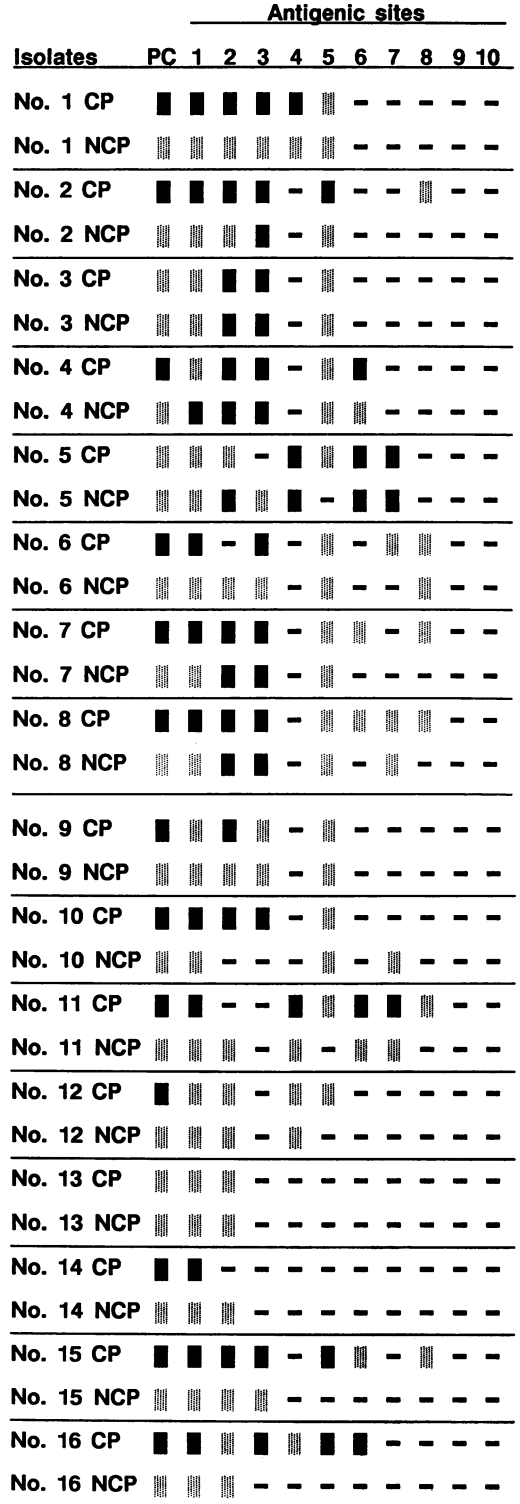
Lack of antigenic variation over time. CP BDV and NCP BDV isolates taken from a single herd of cattle with recurring episodes of BDV infections were tested for their reactivity with the MAbs to examine the effect of time on

TABLE 3. Antigenic variability of a cloned NCP BDV isolate[a]

Isolates	Antigenic sites										
	PC	1	2	3	4	5	6	7	8	9	10
Uncloned	▒	▒	■	■	▒	-	-	-	-	-	-
11 Clones	▒	▒	■	■	▒	-	-	-	-	-	-
6 Clones	▒	▒	■	■	■	-	-	-	-	-	-
2 Clones	▒	▒	■	■	-	-	-	-	-	-	-
1 Clone	▒	▒	■	■	■	-	-	-	▒	-	-

[a] See Table 2, footnote *a*, for definitions.

TABLE 4. Antigenic relationship between CP and NCP pairs of viruses from fatal cases of BDV infection



[a] See Table 2, footnote *a*, for definitions.

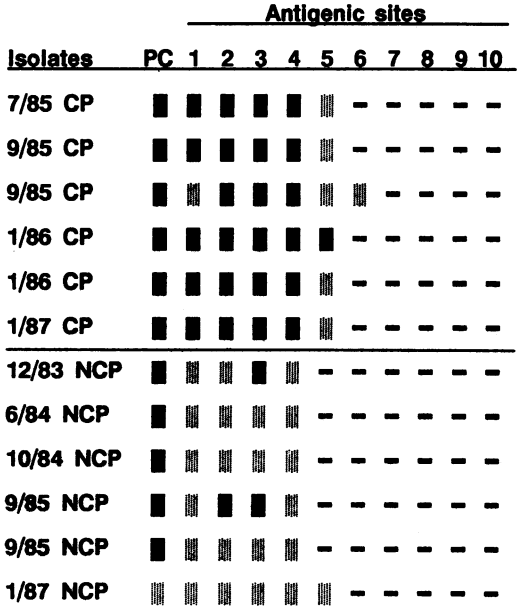
antigenic variation. A total of 12 different CP and NCP isolates taken from 11 different animals over a 3-year period, from December 1983 to January 1987 were examined. The patterns of antigenic sites present were nearly identical among all the isolates within each biotype (Table 5). In addition, the antigenic patterns between the two biotypes were also very similar, varying at only one site in most instances (site 5). The CP BDV and NCP BDV isolates taken from this farm in January 1987 were both derived from the same animal (also shown in Table 4, case 1). All other isolates were taken from different animals.

DISCUSSION

The mechanism by which NCP BDV is maintained in apparently healthy infected cattle is believed to be a specific immunotolerance of persisting NCP BDV. Persistently infected cattle are capable of mounting an immune response to other viral infections and, in many instances, to experimental challenge with some strains of CP BDV (1, 12; unpublished observation). In the majority of natural cases, however, persistently infected cattle lack serum neutralizing and nonneutralizing antibodies to BDV. This specific immunotolerance of the persisting virus is not lost, even in the presence of superinfection with CP BDV (6). The evidence strongly supports the view that in superinfections resulting in fatal disease, CP BDV is antigenically identical or closely similar to persisting NCP BDV.

Using a panel of 22 MAbs that recognize at least 10 different antigenic determinants on the two major glycoproteins of BDV, we have demonstrated that a high degree of antigenic similarity exists between CP and NCP pairs of viruses capable of causing fatal disease in cattle. Although the degree of similarity between CP and NCP biotypes within a pair varied slightly among different pairs of viruses (Table 4), it nonetheless stood in marked contrast to the wide

TABLE 5. Antigenic variability of CP BDV and NCP BDV within a herd[a]



[a] See Table 2, footnote *a*, for definitions. Numbers in the stub represent month/year.

range of variability that existed among unrelated viruses of either biotype (Table 2). CP BDV and NCP BDV pairs from natural infections tended to vary at no more than 3 of the 10 antigenic sites examined, and in more than one third of the cases the two biotypes within a pair were indistinguishable from one another. Unrelated isolates of either biotype varied to some degree at all antigenic sites examined (Table 2). Isolates that contained an extremely diverse range of possible combinations of antigenic sites were found, demonstrating the high degree of antigenic variability of BDV.

Antigenic similarity was less evident in pairs resulting from experimental or vaccine challenges of persistently infected cattle with CP BDV. The two cases examined varied at 3 and 4 of the 10 antigenic sites (Table 4, cases 15 and 16). A certain degree of dissimilarity between isolates of the two biotypes capable of causing fatal disease is therefore possible but is not present in most natural infections. The exact limits of this dissimilarity are not known, but the fact that such limits exist is clear from multiple challenge experiments of persistently infected cattle with different CP BDV isolates (unpublished observation).

In natural infections, the origin of CP BDV is almost certainly a mutation from NCP BDV replicating within persistently infected cattle. The close antigenic similarity between the two biotypes isolated from fatal cases of BDV infections does not support any other conclusion. The spontaneous mutation frequency of RNA viruses is quite high and has been estimated to be somewhere between 10^{-3} to 10^{-4} mutations per incorporated nucleotide (9). Cattle persistently infected with NCP BDV harbor virus in almost all tissues of the body, and infectious virus can be isolated from most bodily secretions. With such a high mutation frequency and such a large pool of replicating virus, it is intriguing that CP BDV is not expressed even more frequently in these animals than it is. Mutation to the CP biotype may, therefore, require two or more independent mutations, a process that would significantly decrease the frequency of its occurrence. With such a mutation having once occurred, rapid transmission to other persistently infected cattle harboring the same strain of NCP BDV could produce explosive outbreaks of fatal disease.

Mutations that alter the reactivity patterns but not the biotypes of individual isolates frequently occur, and variants can be selected by their resistance to neutralization by neutralizing MAbs (W. V. Corapi and E. J. Dubovi, unpublished observation). It is somewhat surprising, therefore, to find a high degree of antigenic stability of BDV within a herd of cattle over a significant length of time. All six CP BDV and six NCP BDV isolates taken from a single herd of cattle over a 3-year period have maintained reactivity patterns that are almost indistinguishable from one another (Table 5). Replication of the parental virus in persistently infected cattle must, therefore, be favored over that of new variants. Variant expression may also be influenced by the production of low levels of antibody that could select against new variants.

Whatever factors govern the selection of CP BDV from NCP BDV, they do not necessarily influence the antigenic makeup of the virus, since isolates of both biotypes taken from the same animal and with identical patterns of reactivity with the MAbs could be found (Table 4). Since persistently infected cattle are capable of responding immunologically to experimental challenge infection with some strains of CP BDV while remaining immunotolerant of persisting NCP BDV, the conservation of antigenic sites within the persisting virus is most likely favored over antigenic variation in these animals. These findings support our belief that antigenic variation and biotype are two independent variables. The molecular basis for the property of cytopathogenicity appears to be dependent on the posttranslational processing of the 118-kDa viral protein to 80 kDa (7), while the antigenic variation that we have observed with the panel of MAbs is a result of differences in the 56- to 58-kDa glycoprotein of the virus (4).

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