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BVD Virus Antigens in Tissues of Persistently Viraemic, Clinically Normal Cattle: Implications for the Pathogenesis of Clinically Fatal Disease

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Bielefeldt Ohmann, H.: BVD virus antigens in tissues of persistently viraemic, clinically normal cattle: Implications for the pathogenesis of clinically fatal disease. Acta vet. scand. 1988, 29, 77-84. – The cellular events involved in precipitation of the clinically fatal outcome of an infection with bovine viral diarrhoea virus (BVDV) remain unresolved, though it is now known that this course of the infection, Mucosal Disease (MD), only occurs in calves persistently infected with non-cytopathic BVDV. In studies aimed at elucidating the pathogenesis of MD, the distribution of BVDV antigens and infectious virus in tissues of persistently infected, clinically normal calves was investigated. Virus antigen was detected in most tissues, in epithelial and immune cells. No signs of an inflammatory response were detected and cytopathological changes were subtle or absent. The infection may nevertheless create a cell-environment which will enhance replication of cytopathic virus. Variations in the clinical, pathomorphological and virological appearance of MD-cases may depend on both the host-reactions, including virus-induced immunopathology, and the virus-strain combinations in a putative mixed infection.

bovine viral diarrhoea virus; persistent infection; tissue distribution; immunocytochemistry, viral pathogenesis.

Introduction

There is now good evidence to suggest that it is only cattle persistently infected with non-cytopathic (ncp) Bovine Viral Diarrhoea virus (BVDV), following an in utero infection in the first trimester of gestation, that become afflicted by the clinically fatal disease, known as Mucosal Disease (MD) (*Duffell & Harkness* 1986). Moreover, the indications are that precipitation of clinical MD follows superinfection with a cytopathic (cp) BVDV strain. The latter may be encountered by transmission (*Barber et al.* 1985) or arise endogenously as a result of genomic mutation in the persisting ncp virus strain (*Brownlie et al.* 1986). Both theories, not mutually exclusive, however, leave several questions open. For example, if exogenous in nature, what is the reservoir of the cp-strain(s)? And what is, if any, the significance of antigenic relationship between the cp-strain and the persisting virus strain? Considering the mutation rate of RNA viruses in general (*Holland et al.* 1982), why does a stable cp strain in the majority of cases not emerge and cause disease until the calf is ½ yr or older? Is the putative mutation dependent on a specific maturational stage in a particular cell type(s) or organ system, perhaps governed by hormonal changes during puberty (*Roeder & Drew* 1984)? Or is it regulated by immunological mechanisms, yet to

be disclosed? It is known from other virus-host systems that the host cell type can influence the properties of the emerging virus, whether it be its cell tropism, antigenic components or virulence (*Rager-Zisman et al.* 1984; *Candurra & Damonte* 1985; *Taguchi et al.* 1985). Thus, it seemed pertinent to the elucidation of the pathogenesis of MD to establish the identity of cell(s) harbouring and replicating the virus in persistently infected, clinically normal cattle. It was recently shown that the infection occurs in T- and B-lymphocytes, monocytes as well as null (non-T, non B-, non-macrophage) cells in peripheral blood of such animals (*Bielefeldt Ohmann* 1987; *Bielefeldt Ohmann et al.* 1987). In this report, the results of virological examination of all tissues in persistently infected, healthy calves are described.

Materials and methods

Calves and young adults in clinically healthy condition were identified as persistently viraemic by repeated virus isolation from purified blood mononuclear leukocytes and plasma (*Bielefeldt Ohmann et al.* 1987). The animals all remained healthy and negative for BVDV-specific serum-neutralizing antibodies up until the time of slaughter or euthanasia (6-22 months of age). Tissue samples were collected aseptically and processed for virus isolation (*Bielefeldt Ohmann et al.* 1987), immunocytochemistry (*Bielefeldt Ohmann* 1987) and light and electron microscopy (*Bielefeldt Ohmann & Bloch* 1982). Nasal swabs were collected on several occasions from calves, without (n = 4) or with (n = 6) an experimentally established BVDV viraemia, all housed together (*Bielefeldt Ohmann et al.* 1987). Smears made from these were examined for BVDV antigen by immunofluorescence (*Bielefeldt Ohmann* 1987) and virus isolation from swabs was attempted as described elsewhere (*Bielefeldt Ohmann et al.* 1987) using BVDV-free MDBK cells. A tissue or swab-sample was not declared virus-negative until after the fifth passage without positive antigen detection by immunoperoxidase staining (*Bielefeldt Ohmann et al.* 1987). For the immunocytochemical detection of virus antigen, tissue sections and cytospin preparations from animals negative for BVDV by isolation were included as specificity controls (*Bielefeldt Ohmann* 1987; *Bielefeldt Ohmann et al.* 1987). For analysis of virus-induced polypeptides by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotting, MDBK cell cultures were infected with virus isolates from animals in this study, or with Danish cytopathic and non-cytopathic BVDV stains, isolated from clinical material and cloned by limiting dilution. After 48 h of incubation the cell cultures were washed, solubilized (50 mmol/l TRISMA-base, 150 mmol/l, 1 mmol/l, 0.5% Na-Deoxycholate, 0.1% Na-dodecylsulphate, 1% NP40, 0.1% Na-azide, 0.1% gelatine and 10^4 i.e./l of aprotinin, pH 8.0) mixed with 20% sample buffer, heated 1 h at 56°C and electrophoresed on 10% SDS-polyacrylamide gels according to *Laemmli* (1970). This was followed by electro-blotting to nitrocellulose membranes (0.45 µm, Schleicher and Schuell, FRG) for 2 h at 4 V/cm as described by *Towbin et al.* (1979). A molecular weight standard from BIO-RAD was included in each gel. After blocking for 5 min with 2% Tween 20 in PBS, the virus specific antigens were detected with a bovine hyperimmune serum against BVDV (courtesy of Dr. K. Dalsgaard) by incubation for 2 h, 38°C, followed by HRP-conjugated rabbit-anti-bovine Ig (DAKOPATTS) for ½ h, 38°C, and the substrate (0.05% DAB, 0.015% H_2O_2 in 0.1 mol/l PBS, pH 7.2). The membranes were washed thrice in PBS with 0.02% Tween 20 between incubation steps.

Results

The results of studies on BVDV isolation and antigen detection in persistently viraemic, clinically normal cattle are presented in Table 1. In all cases the virus strains isolated were non-cytopathic for MDBK cells. Virus induced proteins from some of these isolates were analyzed by SDS-PAGE and electroblotting after 1 or 2 passages of the virus in MDBK cells. For comparison cp virus strains, isolated from cases of MD and clone-purified by limiting dilution, were assayed in parallel. These investigations revealed 6-8 virus induced polypeptides expressed in the susceptible MDBK cells, with molecular weight of approximately 165, 135, 120, 110, 92, 80, 56, 48 and 27 kD, respectively, with some variation between virus-isolates in the magnitude of protein expression. Notably, a polypeptide with a molecular weight of approximately 80 kD, characteristically expressed by cytopathic BVDV-strains, was always lacking in the noncytopathic virus isolates from the clinically healthy calves (Fig. 1). In contrast, some of the cytopathic strains lacked the 110 and 85 kD polypeptides (fig. 1 and data not shown). In some isolates two polypeptides of 118-120 kD could be discerned. BVDV was isolated from nasal swabs from most of the persistently infected calves, even though gross contamination with blood was absent. BVDV was also on some occasions isolated from the swabs of in-contact animals, otherwise characterized by having serum-neutralizing antibodies and no detectable virus (or virus antigen) in the blood (ie. in leukocytes or plasma). However, only in the former group was BVDV antigen detected in cells on smears, indicating that the latter isolates most likely originated from virus-contamination of the barns, either by aerosols or saliva (*Duffell & Harkness* 1985). Virus-positive cells in the nasal smears of persistently viraemic cattle were

Table 1. BVDV isolation and antigen detection in persistently infected, clinically normal cattle.

Tissue	Virus-Isolation	Virus-Antigen[d]	Cell-Type(s)[e]
lymph nodes[a]	+	+ + + +	lymphocytes, macroophages
ileum	+	+ +	lymphoid cells, epithelial cells (few)
colon	+	+	lymphoid cells
rumen	NT[c]	+ +	keratinocytes
omasum	NT	+ +	keratinocytes
abomasum	NT	+ +	mucosal epithelial cells 1. propria cells
oesophagus	NT	+ +	keratinocytes
lungs	+	+	bronchiolar epithelium
gingiva/labia	+	+ +	keratinocytes
tongue	NT	+ +	keratinocytes
skin[b]	+	+ +	keratinocytes
brain	+/-	+	microglia (?)
thymus	+	+ + +	thymocytes, macrophages, IDC
spleen	+	+ + +	lymphocytes, macrophages

[a] Bronchial, cervical and mesenterial lympho nodes tested separately, but with identical results.

[b] Skin biopsies taken from different sites, including interdigital cleft.

[c] Not tested.

[d] Virus antigen load graded subjectively from 0 to 4+.

[e] The predominant cell type(s) containing virus antigen.

identified as either epithelial cells (ciliated) or mononucelar leukocytes. In all tissues examined, the cells infected with BVDV appeared to be of the same types as those observed in calves succumbing to MD (*Bielefeldt Ohmann* 1983), ie., keratinocytes in str. basale and str. spinosum of the skin, of tonsillar crypts and of the upper digestive tracts; asinar cells of salivary glands; bronchiolar epithelial cells; tubular epithelial cells of the kidneys; a proportion of histiocytes in most tissues, including Kuppfer cells in the liver and finally, in lymphocytes, macrophages and dendritic cells of the lymphoid tissues. Only in the extent, ie., numbers of infected cells and/or antigen-staining intensity, did the healthy calves differ from MD calves. This was most notable in the gut, where only few cells in the base of the crypts were infected in the former group (Fig. 2), whereas the infection often spans the entire depth of the crypts in MD-calves (*Bielefeldt Ohmann* 1983).

Moreover, in the gut material examined so far, epithelial infection has not been detected beyond the ileocaecal orifice. Below this anatomical site the intestinal infection was confined to intraepithelial lymphocytes and mononuclear cells in lamina propria. In the brain of clinically healthy calves only cells tentatively identified as microglia cells (or small neurons; differentiation not possible in cryosections) contained viral antigens, whereas widespread infection of large neurons occurs in MD calves (unpublished data). Finally, as there were no evidence of pathological lesions or inflammatory reactions in tissues of the healthy, persistently infected calves, apart from incidentially encountered small interstitial infiltrates of mononuclear cells (in kidneys, liver triads and musles), these animals lacked the infection component contributed by cells in such inflammatory cell accumulations, as typically seen in MD-calves (*Bielefeldt Ohmann* 1983, 1987). Electron microscopic examination of a number of tissues, including intestines, lymph nodes and thymus, did not reveal any significant cytological changes (not shown).

Discussion

The present investigation notably demonstrated that despite widespread occurrence of virus-antigen and infectious virus in most tissues, representing a variety of different cell types, including epithelial cells of keratinized and mucosal surfaces, lymphocytes and macrophages, neither cytopathology nor an inflammatory response was evident in the clinically normal animals. These findings are in general agreement with case reports and experimental works by others (*Barlow et al.* 1986, *Hewicker et al.* 1987; *McClurkin et al.* 1984), and suggest that the immunotolerant state involves not only the antibody response but also cell mediated (T-cell dependent) mechanisms (*Scott* 1984).

Glomerulonephritis caused by antigen (BVDV)-antibody deposition, has previously been reported to occur in some persistently infected, clinically normal cattle (*Coria & McClurkin* 1978; *Bolin et al.* 1985b; *Cutlip et al.* 1980); however, this condition was not detected in the present material, neither has it been seen in our previous studies of calves succombing to MD (*Bielefeldt Ohmann* 1981, 1983). Thus, its general significance in the MD-syndrome may be disputed as also speculated by others (*Hewicker et al.* 1987). In contrast, we have on many occasions observed changes in the structure of keratinized epithelia, especially in the upper part of the digestive tract, comprising hypoplasia of the epithelium and/or exaggeration of the rete pegs/subepithelial ridges without concomittant frank lesions. This could be taken as an indication of altered growth and differentiation of the epithelium due to a ch-

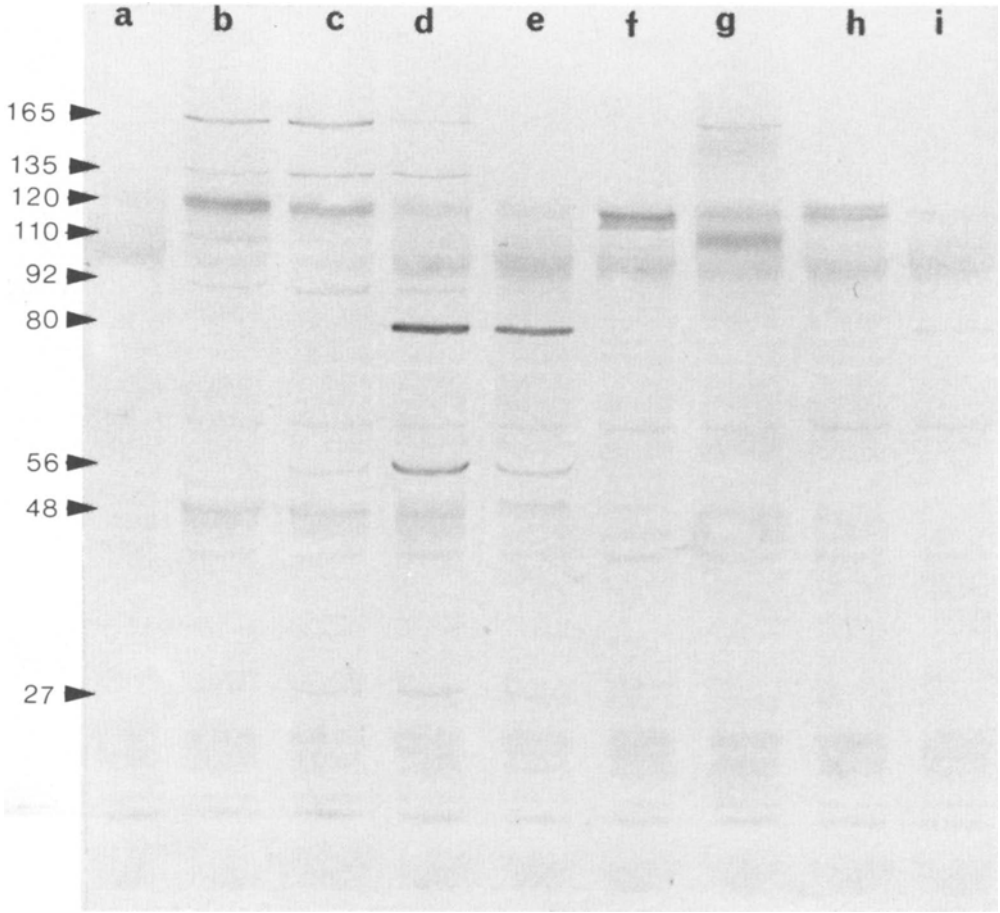

Figure 1. »Western blot« analysis of BVDV-specific proteins expressed in MDBK cells. Lane a: Mock-infected MDBK cells; lane b: BVDV isolate from a macrophage culture established from blood monocytes of a persistently infected healthy calf; lane c: BVDV isolated from PBL of a persistently infected healthy calf; lane d: BVDV isolated from ileum of a persistently infected calf, which succumbed to MD; lane e: BVDV strain Ug59 (cytopathic); lane f: Danish non-cytopathic BVDV strain (isolate 9858); lane g: BVDV isolated from cell-free plasma of a persistently infected healthy calf; lane h: Danish non-cytopathic BVDV strain (isolate 10039); lane i: Danish cytopathic BVDV strain (isolate 10073). Mock-infected or virus-inoculated MDBK cell cultures were harvested 24-48 h p.i. and applied to SDS-PAGE and electroblot to nitrocellulose membranes. The viral proteins were revealed by immunolabelling with a bovine BVDV-specific hyperimmune serum and a horse radish peroxidase conjugated rabbit anti-bovine antibody followed by DAB-substrate. Due to the low contrast of this staining method not all of the bands may be discerned on the photograph. Molecular weights are shown, x 10^{-3}.

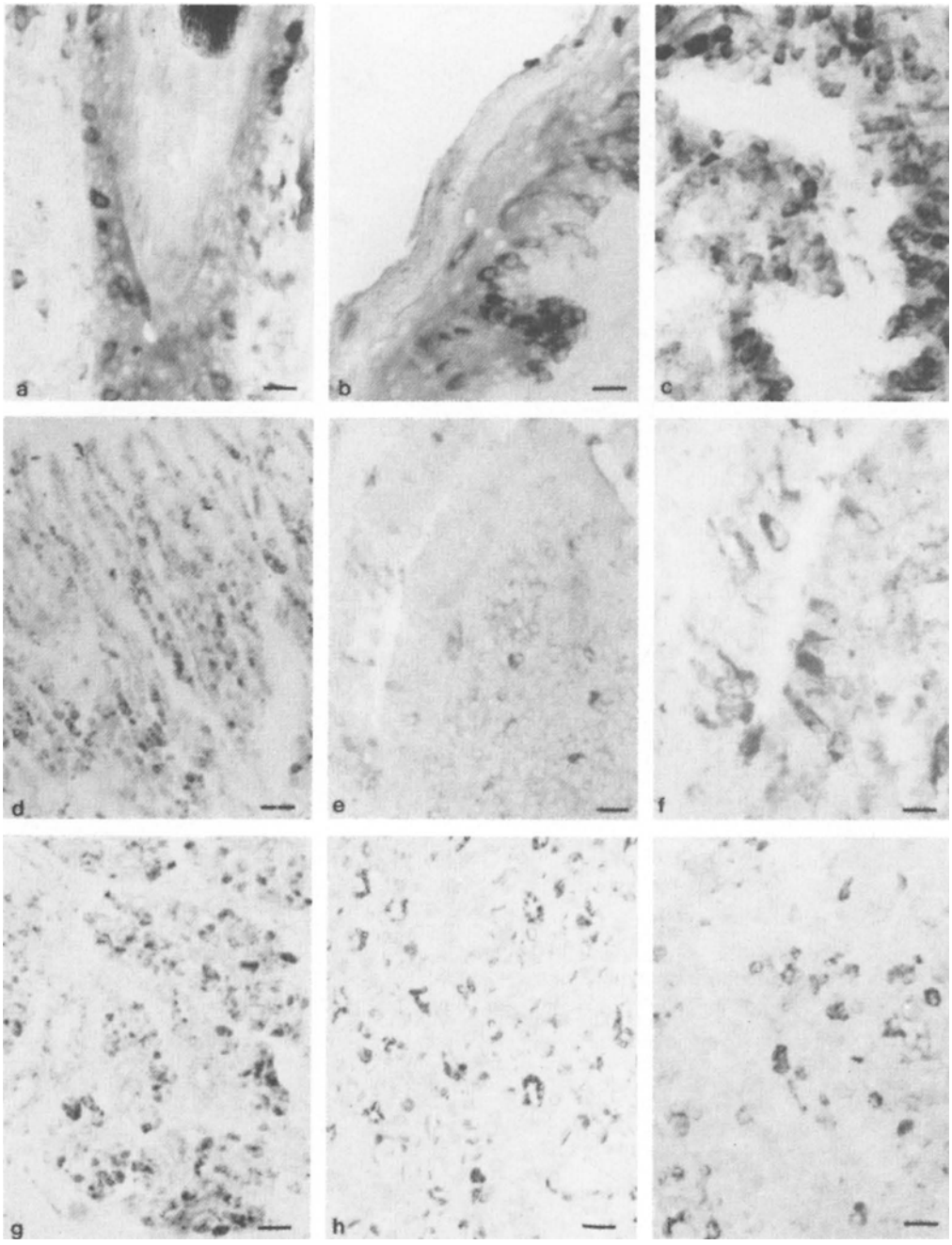

Figure 2. Immunoperoxidase staining of cryostat sections of tissues from persistently viraemic BVDV-infected, clinically normal calves. No histomorphological lesions were found in the tissues. (a) Virus-positive cells in basal layer of a hairfollicle and scattered BVDV-positive. Mø-like cells in dermis. Bar = 40 um. (b) BVDV positive cells in epithelium of oesophagus. Bar = 40 um. (c) Intense antigen-reaction in bronchiolar epithelium in lungs. Bar = 40 um. (d) Cells positive for BVDV antigen among mucosal epithelial cells in abomasum. Bar = 100 um. (e) Scattered BVDV positive cells in Peyer's patches of the intestines. Bar = 40 um. (f) BVDV antigen containing cells in mucosal epithelial cells of ileum. Bar = 25 um. (g) Salivary gland epithelial cells show strong positive reaction for BVDV antigens. Bar = 100 um. (h) Widespread BVDV infection of tubular cells in the kidneys. Bar = 100 um. (i) BVDV positive cells in the cortico-medullary zone of thymus. Bar = 40 um.

chronic adverse effect of viral replication (*Yoneda et al.* 1986). BVDV is known to cause growth retardation (*Done et al.* 1980), and may also cause other subclinical pathological conditions prior to fulminant MD (*McClurkin et al.* 1984). Such findings bring into focus the questions concerning the temporal cause-effect relationship between cytopathological changes, clinical disease and the demonstrable occurrence of cytopathic virus, if indeed the latter is always necessary in order for MD to develop (*Roeder & Drew* 1984; *Littlejohns et al.* 1985) (vide infra).

Several scenarios for the pathogenesis of fullminant MD could be envisaged. An impaired immune response, as expressed by virus-specific immunotolerance, might allow an antigenically closely related cytopathic virus strain to infect, replicate, spread and destruct tissues (*Brownlie et al.* 1984; *Bolin et al.* 1985a), perhaps followed by exacerbation due to a non-specific inflammatory response. Another theory suggests that a cytopathic BVDV might, due to antigenic differences, actually stimulate the immune response and this could, due to cross-reacting antigens eventually lead to an autoimmune-type disease process (*Bolin et al.* 1985b; *Sharpe & Fields* 1985).

None of these hypotheses, however, directly furnish an explanation to the question about the source of the cytopathic virus, although they are both compatible with the suggestion that the cp strain may arise de novo in at least one animal in a serial outbreak of MD (*Brownlie et al.* 1986).

A third hypothesis, which seems more compatible with the apparent stability of the BVDV genome, at least in vitro, as well as with data from field cases (*Barber et al.* 1985; *Littlejohns et al.* 1985) and also experimental data (*McClurkin et al.* 1984; *Rønsholt* personal communication, *Straver* personal communication), would suggest that at least in some persistently infected animals, the persisting virus-pool is a mixture of cytopathic and non-cytopathic virus. The replication of the cp strain(s) however, is suppressed due to the well-known interference effect of ncp strains (*Diderholm & Dinter* 1966, *Sharpe & Fields* 1985, *Whitaker-Dowling & Youngner* 1987). Replication of the ncp strain will, despite lack of frank cytopathology, cause a slow deterioration of cell functions and repair processes (*Oldstone et al.* 1982, *Yoneda et al.* 1986). This long-term effect alone or in combination with other age-related cell-differentiation/maturation events may in some animals create a cellular mileau which will support replication of the cytopathic virus strain directly or indirectly, by inhibiting the noncytopathic strain (*Whitaker-Dowling & Youngner* 1987). Once this process has started it may be exacerbated by an inflammatory reaction. Virus-induced cytopathic lesions may attract macrophages (*Ward et al.* 1972), which by their secretion of hydrolases and toxic oxygen-species could contribute to the tissue damage in a particular area. At this stage the process may evolve into a circulus vitiosus.

This hypothesis does not exclude the possibility that cytopathic virus can arise de novo in some cases, but it does, on the other hand, imply that this is not a necessary event for MD to occur. Furthermore, the theory does not imply that all persistent infections are of the mixed type. Perhaps only a small number is or, alternatively, only a limited number is of a cp-ncp-combination which will eventually precipitate MD (*Barber et al.* 1985, *Bolin et al.* 1985a, 1985b, *Rønsholt* personal communication, *Straver* personal communication). However, once replication of the cp strain takes over in one animal the virus can spread to other persistently infected animals within a herd (and beyond) and

if given the »right« cn-ncp-combination a series of MD-cases will ensue. A temporal scenario of this type would help explain the variations in both the clinical picture and pathomorphological lesions, as well as the results of virus isolations (*Bielefeldt Ohmann* 1981, *Roeder & Drew* 1984, *Barber et al.* 1985, *Littlejohns & Walker* 1985 and own unpublished data).

Confirmation or refutation of this hypothesis for the pathogenesis of MD may have to await more detailed characterization of the BVDV genome and proteins and the availability of reagents (genomic probes and antibodies) which can be used to detect and distinguish the in situ distribution of non-cytopathic and cytopathic virus, respectively, in persistently infected, clinically normal cattle as well as in animals afflicted by MD.

Finally a comment on the epidemiological implication of the present findings can be made. Virus are likely to be continuously shed in saliva and via aerosols from the persistently infected cattle. Shedding via the urine may also occur (*Mills et al.* 1968) though this was not tested for in the present investigations. In contrast, fecal virus-excretion did not occur in detectable amounts until the animals contracted clinical MD (unpublished data; *Rønsholt* personal communication), a finding in agreement with the distribution of virus-antigen in the intestine of normal and diseases calves, respectively. Taken together, the results do corroborate the notion of persistently infected, clinically healthy animals as an epidemiological threat to the cattle population, and their elimination should be persued.

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