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Identification of cell membrane proteins linked to susceptibility to bovine viral diarrhoea virus infection

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Summary. Three monoclonal antibodies directed against cell surface molecules of bovine cells inhibited subsequent infections with bovine viral diarrhoea virus (BVDV). They specifically blocked the infectivity of three non-cytopathogenic and three cytopathogenic BVDV strains. These results showed that an important mechanism for virus uptake was inhibited. The ligand of the monoclonal antibody BVD/CA 17, which blocked infectivity most efficiently, was found on leukocytes from a wide range of domestic and wild even-toed ungulates using flow cytometric analysis. In contrast, the monoclonal antibodies BVD/CA 26 and BVD/CA 27 appeared to be specific for bovine cells. Immunoprecipitation of labelled bovine cell surface proteins showed that the three monoclonal antibodies bound to proteins with identical relative molecular masses (M_r). Proteins of an apparent M_r of 93 K and 60 K were precipitated from lysates of fetal bovine kidney cells irrespectively of the MAbs used.

Introduction

Pestiviruses are enveloped, plus-strand RNA viruses which comprise pathogens of great economical importance for the farming industry. They are serologically closely related and infect pigs, sheep, cattle and a wide range of wild living even-toed ungulates [29]. The bovine viral diarrhoea virus (BVDV) is the prototype virus of the genus Pestivirus and induces a variety of syndromes in cattle ranging from inapparent infections to fatal mucosal disease (MD) [2, 28]. Two biotypes of BVD virus can be distinguished according to cytopathogenicity in cultured cells. Both the non-cytopathogenic and the cytopathogenic biotype can be isolated from cases of MD [25]. BVD virus has a marked tropism for the epithelial cells of the respiratory and enteric tract of its host [2, 3] and infects lymphoid cells [5, 6, 12, 23, 35] as well as other susceptible target cells [1, 4, 10, 13, 17].

The early phases of viral infection are of obvious importance for the understanding of cell tropism and pathogenesis. For BVD virus little is known about the molecular components of susceptible bovine cells and their viral counterparts which are responsible for virus binding and subsequent uptake of virus particles. Xue and Minocha [38] identified a single cell surface molecule with an M_r of approximately 50000 (50 K) on Madin-Darby bovine kidney (MDBK) cells which is probably involved in binding of the viral envelope protein gp 53. The viral glycoprotein gp 53 is the major target for neutralizing monoclonal antibodies (MAbs) [7, 11] and it forms covalently linked homodimers as well as heterodimers with gp 25, the second glycoprotein present in the viral envelope [34, 37]. Recently, Boulanger et al. [8] showed that the third envelope glycoprotein gp 48 also induces neutralizing MAbs. This supports the assumption that both major envelope proteins, gp 53 and gp 48, are involved in attachment to the cell surface or penetration of the host cell.

In this communication we extend a previous report [27] using three MAbs directed against cell surface components of bovine cells which block virus infection. The experiments presented in this paper were conducted to better characterize host surface components that are involved in virus binding or virus entry. In order to determine whether the three MAbs BVD/CA 17, BVD/CA 26 and BVD/CA 27 were suitable for the identification of a BVD virus receptor, we tested the ability of the MAbs to inhibit infection of cultured cells by different BVDV strains, their binding activity to leukocytes of different species and identified cell surface molecules responsible for binding of the MAbs.

Materials and methods

Cells and viruses

Primary fetal calf kidney (FCK) cells were prepared as described [30]. FCK cells were grown in Dulbecco's modification of Eagle's minimal essential medium (EDULB) supplemented with 5% BVDV free bovine serum, 100 units/ml penicillin-G and 0.1 µg/ml dihydrostreptomycin. Cultures were tested for the absence of BVDV antigen by a peroxidase-linked antibody (PLA) binding test.

The cytopathogenic BVDV strains NADL, TGAC, and Indiana as well as the non-cytopathogenic strains Nebraska, Auburn and 7443 originated from the National Animal Disease Center, Ames, USA. Viruses were propagated in FCK cells and stocks were stored frozen at –80 °C. Virus titers were determined as described [19].

The bovine herpes virus type 1 (BHV-1), strain Colorado 1, and parainfluenza 3 virus field isolates used as negative controls for virus inhibition assays originated from the Institute of Virology, Hannover Veterinary School, Hannover, FRG.

Monoclonal antibodies

For production of MAbs bovine turbinate (BT) cells were infected with the cytopathogenic strain NADL. For immunization of mice, BVD virus was concentrated from cell culture supernatant by ultracentrifugation according to Peters et al. [31]. Due to the strong association of BVD virus to cellular membranes the preparation used for immunization was crude material containing viral as well as cellular antigens. Hybridoma production and

screening for hybridoma clones which produce MAbs with neutralizing activity to BVD virus was done as described by Bolin et al. [7]. Binding of MAbs to non-infected bovine cells was tested in a monolayer enzyme immunoassay [14]. MAb FF/C 35 directed against *Francisella* sp. [15] and MAb BVD/C 16 directed against the non-structural protein p125 of BVDV [31] were used as control antibodies.

Antibodies were purified by affinity chromatography on a goat-anti-mouse-IgG Sepharose 4B column following the instructions given by the manufacturer (Pharmacia, Freiburg, FRG).

Inhibition of virus infection by MAbs

Secondary or tertiary FCK cells were seeded into microtiter plates (1.5×10^5 cells/ml) and incubated overnight in EDULB containing 5% bovine serum. Serial twofold dilutions of MAbs were prepared ranging from 5 µg/ml to 78 ng/ml. Cells were treated for 30 min with 100 µl of each antibody dilution and thereafter 100 µl virus suspension (10 000 $TCID_{50}$/ml) was added. After 44 h incubation at 37 °C cells were washed twice for 3 min each with phosphate buffered saline (PBS, dilution 1:3 in H_2O) and fixed for 2 h at 80 °C. Unspecific binding was reduced by incubation with 0.4% instantized dry milk in PBS/0.02% Tween-80 for 1 h at 37 °C. After three washes for 3 min with PBS/0.02% Tween-80 an anti-BVDV peroxidase conjugate [19] diluted 1:400 in PBS containing 0.2% instantized dry milk and 0.02% Tween-80 was added for 1 h. The plates were washed and the reaction with substrate (H_2O_2/ABTS; 2,2′-azino-bis [3-ethylbenzthiazolin]-6-sulfonic acid) was measured colometrically after 40 min at room temperature at a wavelength of 405 nm (ELISA-reader SLT Spectra II, SLT Labinstruments, Austria). All titrations were done in triplicate. Cells infected with BVDV without previous incubation with MAbs and non-infected cells were used as standards to calculate the percentage of inhibition of infectivity according to the formula:

$$\text{Percentage of inhibition} = 100 - [100 \times (a - c)/(b - c)]$$

a: the mean $OD_{405\,nm}$-value from infected cells previously incubated with MAbs
b: the mean reading from infected cells without previous incubation with MAbs
c: the mean reading from non-infected cells

In previous experiments titration curves were established for all BVDV strains used in this study to determine the optimal $TCID_{50}$ value for this test. For all strains 10000 $TCID_{50}$/ml were within the linear range of the slope.

Flow cytometric analyses and immunofluorescence staining of blood leukocytes and FCK cells

Blood from humans, swine (German landrace), rabbits, horses and various domestic ruminants like cattle (Brown swiss and Simmentalian breed), sheep (Merino and Kamerun sheep) and goats (German coloured and African dwarf) was collected after venipuncture in tubes coated with EDTA as an anti-coagulant (Greiner, Frickenhausen, Germany). Blood of wild even-toed ungulates was taken from several captive animals (Zoo Hellabrunn, München, Germany), i.e. alpaka (*Lama vicugna pacos*), ibex (*Capra ibex*), nilgai antilope (*Bosalephus tragocamelus*), bontebok (*Damaliscus dorcas*), and roe deer (*Capreolus capreolus*). Erythrocytes of the EDTA-stabilized blood were lysed with ammonium chloride buffer (8.29 g/l NH_4Cl, 1.0 g/l $KHCO_3$, 0.372 g/l Na_2EDTA) for 10 min on ice and after two washes with cold PBS leukocytes were incubated with hybridoma culture supernatant from BVD/CA 17 or BVD/C 16 (negative control) for 30 min on ice. The cells were washed two times with cold PBS and fluorescein isothiocyanate (FITC) labelled sheep anti-mouse IgG (Sigma, Deisenhofen, Germany) was added to the cells at a final dilution of 1:100 in PBS for

30 min on ice. After two additional washes in cold PBS propidium iodide (1 μM final concentration) was added and samples were analyzed on a FACscan (Becton Dickinson, Heidelberg, Germany). Only samples with more than 90% cells negative for propidium iodide staining were used. Five thousand viable cells per sample were examined. Granulocytes, monocytes and lymphocytes were gated by forward- and sidescatter lights. The FITC-fluorescence analyses were performed by histogram statistics of the Facsscan software. For indirect immunostaining suspended FCK cells and bovine leukocytes were treated as described above, but finally spread on microscope slides and dried. The slides were mounted in PBS/10% glycerol under a fluorescence microscope (Zeiss Axioplan). Excitation of fluorescence was at 450 to 490 nm and for detection a 515 to 565 nm filter was used. Photographs were taken on Kodak Tmax films using a Zeiss 40 × Plan Neofluar objective and a 10 × ocular.

Labelling of cell surface proteins

Cell surface proteins of living cells were biotinylated according to von Boxberg et al. [36]. Secondary or tertiary FCK cells were seeded into 75 cm^2 tissue culture flasks at a concentration of 5×10^5 cells in 20 ml Eagle's minimal essential medium (EMEM) supplemented with 5% bovine serum. After 3 days of culture cells were washed three times with labelling buffer (8.0 g NaCl, 0.4 g KCl, 1.0 g glucose, 0.2 g $MgSO_4.7H_2O$, 0.2 g $CaCl_2.2H_2O$, 0.5 g $NaHCO_3$, distilled water to 1000 ml, pH 7.4) and scraped from the bottom of the flask using a rubber policeman. Resuspended cells were concentrated by centrifugation (800 × **g**, 5 min, 20 °C) and washed again three times with labelling buffer. The final sediment was suspended in labelling buffer at a concentration of 10^7 cells/ml and added to a reaction vial with 20 μl freshly prepared biotin-ester solution (100 mg/ml biotinamidocaproate-N-hydroxysuccinimide ester, Sigma, Deisenhofen, FRG, dissolved in dimethylsulfoxide). After 5 min incubation at room temperature cells were sedimented by centrifugation and washed twice with 1 ml EMEM without serum to remove excess reagent. For immunoprecipitation studies the cells were lysed in 1 ml TNE buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS) containing 0.01% PMSF and 2 μg/ml aprotinin. After 20 min incubation on ice cell debris was sedimented by centrifugation (12 000 **g**, 30 min, 4 °C) and supernatants were stored frozen at −20 °C or immediately used.

Immunoprecipitation and western blotting

Cell lysates were aliquoted (50 μl) and 7 μl of purified monoclonal antibodies diluted in TNE buffer (35 μg/ml final concentration) were added. Lysates were incubated on ice for 1.5 h until antigen-antibody complexes were bound to Protein G-sepharose beads (Pharmacia, Freiburg, FRG) by the addition of 30 μl from a 10% v/v suspension of the beads for 1.5 h. The precipitates were washed five times (3 min, 10 000 **g**, 4 °C) in TNE buffer and the final sediment was resuspended in 50 μl sample buffer (50 mM Tris-HCl, 2% sodium dodecyl sulfate, 50 mM dithiothreitol, 10% glycerol, 0.01% bromophenol blue, pH 6.8). After boiling for 5 min the beads were removed by centrifugation (5 min, 10 000 **g**) and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the discontinuous system devised by Laemmli [20] with 10% separating gels and 5% stacking gels. After SDS-PAGE the proteins were transferred onto polyvinylidendifluorid (PVDF) membranes (Immobilon-P, Millipore, Bedford, USA) for 1 h at 100 V in electrophoresis buffer containing 20% methanol. Membranes were washed briefly in Tris buffered saline (TBS, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5) and incubated overnight at 4 °C in TBS containing 1% blocking reagent (Boehringer Mannheim, Mannheim, FRG). After three washes with TBS containing 0.05% Tween-20 a streptavidin-biotinylated peroxidase complex

(dilution 1:200, Amersham, Braunschweig, FRG) was added. Membranes were incubated for 1 h at room temperature, washed three times for 15 min in TBS/Tween-20 and two times for 3 min in TBS. Membrane bound biotinylated proteins were detected by luminol-based chemiluminescence using western blotting reagents according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, FRG).

Results

Preliminary characterization of MAbs

Screening of hybridoma clones producing MAbs with neutralizing activity to BVD virus revealed fourteen clones. Eleven of these clones produced MAbs which bound to bovine cells infected with BVD virus, but not to non-infected cells. These MAbs were directed against viral proteins as judged by radioimmunoprecipitation experiments [7]. They were not further investigated in this study. The remaining three MAbs BVD/CA 17, BVD/CA 26 and BVD/CA 27 did not recognize viral proteins in radioimmunoprecipitation experiments, but bound to fixed non-infected cells (data not shown). Furthermore, the three MAbs bound to the cell surface of living cells (Fig. 1, data shown for BVD/CA 26 only). Both FCK cells (Fig. 1a) as well as bovine leukocytes from blood samples (Fig. 1b) could be stained in an indirect immunofluorescence assay.

These data suggested that the three MAbs were directed against cellular antigens which were associated to the sedimented virus particles used for immunization of the mice. This close association could be due to a functional involvement of the cellular proteins in virus binding or entry into bovine cells. In order to substantiate this assumption we analyzed the blocking activities of the MAbs and the distribution of their ligands in vivo. In addition, attempts were made in order to identify the target proteins of the MAbs.

Blocking activities of MAbs

The blocking activities of the three MAbs BVD/CA 17, BVD/CA 26 and BVD/CA 27 were measured in the FCK cell system. Three cytopathogenic and

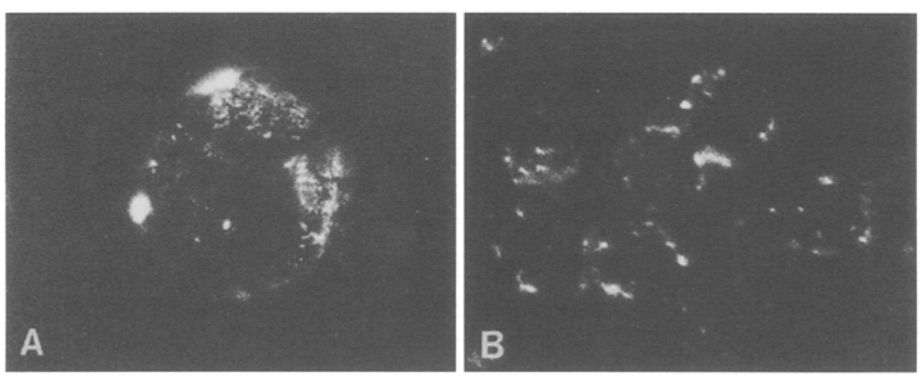

Fig. 1. Indirect immunofluorescence staining of bovine cells. Live FCK cells (**A**) and bovine leukocytes (**B**) were stained with MAb BVD/CA 26 and FITC-conjugated sheep anti-mouse IgG as the secondary antibody

three non-cytopathogenic BVDV strains were chosen for infection to cover a broad spectrum of antigenic variation. The three MAbs effectively blocked the binding of the six BVDV strains tested (Table 1). Among these strains the cytopathogenic strain NADL, which was used for immunization of mice, was completely blocked even with low antibody concentrations. In contrast, the non-cytopathogenic strain Auburn differed from all other strains as complete blocking was not achieved even with high antibody concentrations.

The blocking assay showed that the three MAbs differed in their ability to protect FCK cells from infection with BVD virus. BVD/CA 17 revealed a remarkably higher blocking activity than the two other MAbs. BVD/CA 26 and BVD/CA 27 inhibited virus infection in a dose-dependent manner, whereas BVD/CA 17 had to be diluted to less than 8 ng/well to show this effect. In contrast, infection with non-related viruses (BHV-1 and parainfluenza 3 virus)

Table 1. Inhibition of BVDV infection of FCK cells by MAbs

MAbs	ng/well	Inhibition of virus infection (%)					
		cytopathogenic strains			non-cytopathogenic strains		
		NADL	TGAC	Indiana	7443	Nebraska	Auburn
BVD/CA 17	8	94	92	93	92	85	72
	16	100	100	98	97	98	84
	31	100	100	99	100	100	90
	62	100	100	99	99	100	89
	125	100	99	98	100	99	90
	250	100	100	99	99	100	91
BVD/CA 26	8	21	7	9	33	20	16
	16	46	18	30	26	24	32
	31	88	71	71	69	63	58
	62	100	97	93	93	90	81
	125	100	100	98	100	99	87
	250	100	100	99	100	100	89
BVD/CA 27	8	38	13	22	25	21	24
	16	73	51	64	65	49	47
	31	99	91	89	95	84	76
	62	100	100	97	100	99	86
	125	100	100	99	100	98	86
	250	100	100	97	100	99	87
FF/C 35	8	0	0	0	0	8	6
	16	0	0	0	0	16	4
	31	0	0	0	0	16	2
	62	0	0	0	0	12	10
	125	0	0	0	5	4	10
	250	0	0	0	15	5	14

was not inhibited by the MAbs (data not shown). The control antibody FF/C 35 did not inhibit BVDV infections of FCK cells significantly.

Detection of ligands of MAbs on the surface of living cells from different species

To determine whether the epitopes detected by the MAbs were also present in vivo on the prime target cells of BVDV, blood leukocytes of cattle, sheep, goats and swines were examined by flow cytometry. Figure 2 shows the results obtained with leukocytes from animals of each of the four domestic species. High immunofluorescence staining of all leukocytes was only seen with cattle for all

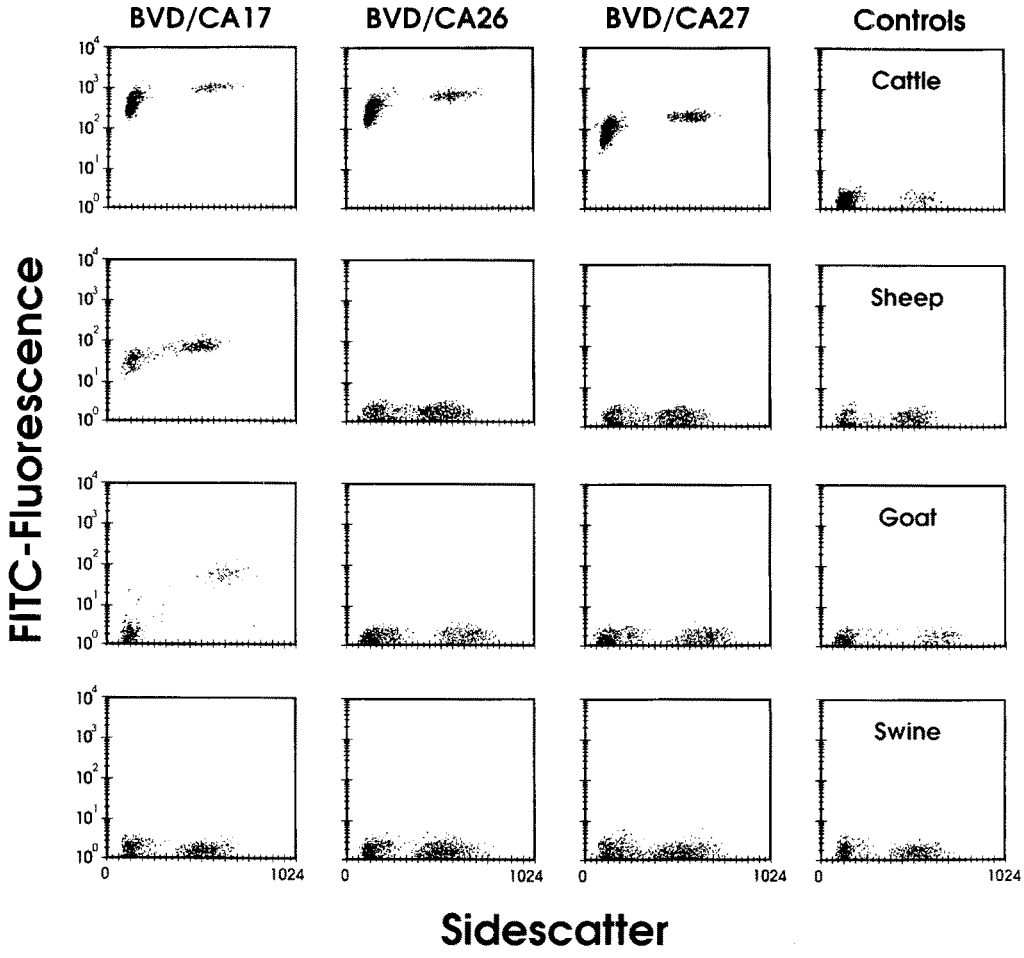

Fig. 2. Detection of ligands of MAbs on the surface of leukocytes of different species by FACS analysis. The X-axis indicates side scatter and the Y-axis denotes FITC-fluorescence intensity. The cluster of dots with high side scatter represents the granulocytes, the left cluster the lymphocytes (left part of the cluster) and monocytes (right part of the cluster). Staining of leukocytes from cattle, sheep, goats and swines (first to fourth row, respectively) was performed with MAbs BVD/CA 17, BVD/CA 26, BVD/CA 27 (first to third lane, respectively) and MAb BVD/C16 (fourth lane) as a negative control for the FITC-conjugate

three MAbs (first row). MAb BVD/CA 26 and BVD/CA 27 did not stain leukocytes from other ruminant species tested so far (lanes 2 and 3, second to fourth row). In contrast, MAbs BVD/CA 17 bound to all leukocytes from sheep (lane 1, second row) and in goats all granulocytes, about 15% of the lymphocytes and approximately half of the monocytes showed a weak staining (lane 1, third row). No binding of MAbs could be detected on non-ruminant leukocytes from swine (fourth row) as well as human, horse, and rabbit (data shown for swine only). In addition, leukocytes from different species belonging to the order *Artiodactyla* were examined. Samples were collected from single captive animals and tested for binding of BVD/CA 17 (data not shown). Immunofluorescence staining of leukocytes from alpaka (*Lama vicugna pacos*) and roe deer (*Capreolus capreolus*) resembled the binding pattern revealed with leukocytes of cattle and goats, respectively. All granulocytes of the nilgai antilope (*Boselaphus tragocamelus*) were stained, whereas lymphocytes and monocytes showed about 70% positive staining. In ibex (*Capra ibex*) only granulocytes showed a positive but weak staining. No staining was observed on leukocytes of the bontebok (*Damaliscus dorcas*). Controls were performed with MAb BVD/C 16 and with conjugate lacking the primary antibody. No staining was observed in these control experiments (lane 4).

Immunoprecipitation of cell surface proteins

Immunoprecipitation analyses were performed to identify the cell surface proteins that were bound by the MAbs. The MAbs did not recognize proteins in western blotting experiments under reducing or non-reducing conditions (data not shown). Therefore, the membrane proteins of FCK cells were labelled with biotin and immunoprecipitated with the three MAbs directed against epitopes on the surface of bovine cells, and with the unrelated MAb FF/C 35. After SDS-PAGE, the proteins were transferred to PVDF-membranes. Biotinylated proteins were detected by chemiluminescence after incubation with a streptavidin-biotinylated peroxidase complex and substrate. The three antibodies showed an identical precipitation pattern of polypeptides with relative molecular masses of 93 000 (93 K) and 60 000 (60 K) (Fig. 3, lanes 2 to 4). Compared to the other MAbs BVD/CA 17 differed in its ability to precipitate proteins, therefore only faint bands were visible in lane 2. The unrelated control antibody FF/C 35 precipitated a different pattern of proteins of high relative molecular masses from the cells (lane 5). In order to visualize even weak bands precipitated by the control MAb, the twentyfive-fold amount of immunoprecipitate was analysed by western blotting. Further, incubation of lysates of non-biotinylated FCK cells with the streptavidin peroxidase complex revealed a single faint band of 66 K (lane 1).

Discussion

Pestiviruses are believed to utilize membranes of the endoplasmic reticulum for their maturation. Mature virus is probably liberated by exocytosis of virus

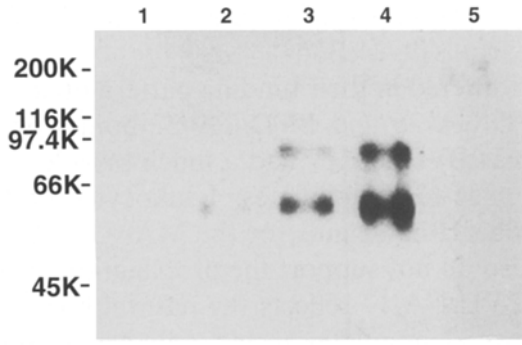

Fig. 3. Immunoprecipitation of biotinylated cells surface molecules from FCK cells with MAbs. Proteins from lysates of biotinylated FCK cells were immunoprecipitated with MAbs BVD/CA 17 (*2*) BVD/CA 26 (*3*) BVD/CA 27 (*4*) or MAb FF/C 35 (*5*) as a negative control. Cell lysates from unlabelled FCK cells were run in *1*. Proteins were detected using streptavidin peroxidase complexes and luminol based chemiluminescence. Bars on the left indicate positions of the marker proteins

containing vesicles [28]. This explains the close association of virus particles and cellular membranes in virus preparations from tissue culture supernatants. Besides non-specific interactions the binding of cellular proteins to virus particles may reflect a functional relationship. Immunization of mice with partially purified BVD virus contaminated with cellular components gave rise to MAbs directed against cellular proteins which may be important in initial stages of BVDV infection.

Our results strongly indicate that the binding sites of the three MAbs are located on or near a major cellular BVDV receptor. By immunofluorescence studies and by immunoprecipitation of biotinylated proteins these antibodies were shown to bind to proteins localized on the surface of susceptible bovine cells. Furthermore, they efficiently blocked virus infection even at low protein concentrations. Inhibition of infectivity was as effective as virus neutralization achieved by MAbs directed against the viral envelope glycoproteins. In addition, it was specific for BVDV, as no protection against infection of cells with unrelated viruses was observed. Each of the MAbs protected FCK cells from infection with cytopathogenic as well as with non-cytopathogenic BVDV strains with comparable efficiency implying that a mechanism crucial for virus infection was inhibited.

Remarkably, inhibition of infection was not always complete, since infection of cells with BVDV strain Auburn was only partially blocked. This finding suggests that pestiviruses may use alternative, but less efficient ways to infect cells. BVD virus strains have a broad host range in vitro and they are widespread contaminants of cultured cells [18, 32]. Although these contaminants seem to comprise mainly non-cytopathogenic BVD viruses which replicate only poorly, some strains do readily grow in ovine and in porcine cells [26]. This indicates that receptor-independent mechanisms of virus entry as well as altered receptor

specificities may play a role in pestivirus infections. Both mechanisms have been described for other viruses [9, 24, 33].

The three MAbs differed in their binding pattern on leukocytes of domestic animals. MAbs BVD/CA 26 and BVD/CA 27 bound exclusively to cells of bovine origin, whereas BVD/CA 17 had a much broader spectrum of binding to leukocytes from even-toed ungulates. Leukocytes of human, rabbit and horse origin did not bear the ligands for the MAbs on their cell surface. Cells from these species also do not support the propagation of BVD virus [18]. The binding pattern of BVD/CA 17 reflects the natural host range of BVD virus. From all wild even-toed ungulates tested only leukocytes of the bontebok showed no staining. Cattle and sheep are highly susceptible to BVD virus infections and leukocytes of both species exhibited high immunofluorescence staining. Infections of goats with BVDV can be induced experimentally, but they do not seem to play an important role in the field. In contrast, inapparent infections of pigs with ruminant pestiviruses are frequently observed [22]. This might support the assumption that pestiviruses are able to vary their receptor specificities.

Multiple attachment sites for BVD virus on bovine cells were proposed after studies performed using anti-idiotypic antibodies [38]. These antibodies were raised against neutralizing antibodies directed against the viral envelope glycoprotein gp 53. Comparing different virus strains and isolates the blocking activities of one anti-idiotypic antibody ranged between 30% and 60% inhibition, implying different attachment sites for different BVDV strains. The molecule which was recognized by the anti-idiotypic antibody was characterized as a cell surface protein of 50 K from MDBK cells.

In contrast, the three MAbs used in this study identified proteins of 60 K and 93 K. As long as there is no information about the identity of these proteins, no statement can be made as to their role for viral attachment and subsequent uptake by the cell. In addition, growing evidence exists that viruses can utilize more than one receptor for attachment and that binding of virions to the cell surface is a multistep event [16, 21].

The three MAbs described in this report will be helpful to identify different steps in early pestivirus infection and to extend our knowledge about mechanisms of neutralization. Further studies will also focus on the purification of the proteins recognized by the MAbs to allow binding studies with viral envelope proteins and biochemical studies to clarify function and relationship of the 93 K and the 60 K protein.

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References

1. Allan GM, McNulty MS, Bryson D, Mackie D, Platten M (1989) Demonstration of bovine viral diarrhoea virus antigen in formalin fixed, paraffin embedded tissue using a streptavidin/biotin technique. Res Vet Sci 46: 416–418
2. Baker J (1987) Bovine viral diarrhoea virus: a review. J Am Vet Med Assoc 190: 242–261
3. Bielefeldt Ohmann H (1982) Experimental fetal infection with bovine viral diarrhoea virus. II. Morphological reactions and distribution of viral antigen. Can J Com Med 46: 363–369
4. Bielefeldt Ohmann H (1983) Pathogenesis of bovine viral diarrhoea mucosal disease: distribution and significance of BVDV antigen in diseased calves. Res Vet Sci 34: 5–10
5. Bielefeldt Ohmann H, Rønsholt L, Bloch B (1987) Demonstration of bovine viral diarrhoea virus in peripheral blood mononuclear cells of persistently infected, clinically normal cattle. J Gen Virol 68: 1971–1982
6. Bolin SR, McClurkin AW, Coria MF (1985) Effects of bovine viral diarrhoea virus on the percentages and absolute numbers of circulating B and T lymphocytes in cattle. Am J Vet Res 46: 884–886
7. Bolin S, Moennig V, Kelso Gourley NE, Ridpath J (1988) Monoclonal antibodies with neutralizing activity segregate isolates of bovine viral diarrhoea virus into groups. Arch Virol 99: 117–123
8. Boulanger D, Waxweiler S, Karelle L, Loncar M, Mignon B, Duduisson J, Thiry E, Pastoret PP (1991) Characterization of monoclonal antibodies to bovine viral diarrhoea virus: evidence of a neutralizing activity against gp 48 in the presence of goat anti-mouse immunoglobulin serum. J Gen Virol 72: 1195–1198
9. Citovsky V, Loyter A (1985) Fusion of Sendai virus or reconstituted Sendai virus envelopes with liposomes or erythrocyte membranes lacking virus receptors. J Biol Chem 260: 12702–12077
10. Corapi WV, French TW, Dubovi EJ (1989) Severe thrombocytopenia in young calves experimentally infected with noncytopathic bovine viral diarrhoea virus. J Virol 63: 3934–3943
11. Donis RO, Corapi W, Dubovi EJ (1988) Neutralizing monoclonal antibodies to bovine viral diarrhoea virus bind to the 56 K to 58 K glycoprotein. J Gen Virol 69: 77–86
12. Ellis JA, Davis WC, Belden EL, Pratt DL (1988) Flow cytofluorimetric analysis of lymphocyte subset alterations in cattle infected with bovine viral diarrhoea virus. Vet Pathol 25: 231–236
13. Greiser-Wilke I, Liebler E, Haas L, Liess B, Pohlenz J, Moenning V (1993) Distribution of cytopathogenic and noncytopathogenic bovine viral diarrhoea virus in tissues from a calf with experimentally induced mucosal disease using antigenic and genetic markers. In: Kaaden OR, Eichhorn W, Czerny CP (eds) Unconventional agents and unclassified viruses. Recent advances in biology and epidemiology. Springer, Wien New York, pp 295–301 (Arch Virol [Suppl] 7)
14. Greiser-Wilke I, Moenning V, Coulibaly COZ, Dahle J, Liess B (1990) Identification of conserved epitopes on a hog cholera virus protein. Arch Virol 111: 213–225
15. Greiser-Wilke I, Soine C, Moenning V (1989) Monoclonal antibodies reacting specifically with *Francisella* sp. J Vet Med B 36: 593–600
16. Haywood AM (1994) Virus receptors: binding, adhesion strengthening, and changes in viral structure. J Virol 68: 1–5
17. Hewicker M, Wöhrmann T, Fernandez A, Trautwein G, Liess B, Moenning V (1990) Immunhistological detection of bovine viral diarrhoea virus antigen in the central

nervous system of persistently infected cattle using monoclonal antibodies. Vet Microbiol 23: 203–210
18. Horzinek MC (1981) Non-arthropod-borne togaviruses. Academic Press, New York, London
19. Hyera JMK, Dahle J, Liess B, Moenning V, Frey HR (1987) Production of potent antisera raised in pigs by anamnestic response and use for direct immunofluorescent and immunoperoxidase techniques. In: Harkness JW (ed) Pestivirus infections of ruminants. Bruessels, Commission of the European Communities, publication CD-NA-10238-EN-C, pp 87–102
20. Laemmli UK (1979) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685
21. Lentz TL (1990) The recognition event between virus and host cell receptor: a target for antiviral agents. J Gen Virol 71: 751–766
22. Liess B, Moenning V (1990) Ruminant pestiviruses infection in pigs. Rev Sci Tech Off Int Epiz 9: 151– 161
23. Lopez OJ, Osorio FA, Kelling CL, Donis RO (1994) Presence of bovine viral diarrhoea virus in lymphoid cell populations of persistently infected cattle. J Gen Virol 74: 925–929
24. Markwell MAK, Portner A, Schwartz AL (1985) An alternative route of infection of viruses; entry by means of the asialglycoprotein receptor of a Sendai virus mutant lacking its attachment protein. Proc Natl Acad Sci USA 82: 978–982
25. McClurkin AW, Bolin SR, Coria MF (1985) Isolation of cytopathic and noncytopathic bovine viral diarrhoea virus from the spleen of cattle acutely and chronically affected with bovine viral diarrhoea virus. J Am Vet Med Assoc 186: 568–569
26. Moenning V (1990) Pestiviruses: a review. Vet Microbiol 23: 35–54
27. Moenning V, Bolin RS, Mateo A, Kelso-Gourley NE, Wilke I, Liess B (1988) Identification of a single receptor for bovine viral diarrhoea virus (BVDV) on the surface of cultured cells. In: Abstracts of the annual meeting of the American Society for Microbiology, Miami Beach, Florida, 8–13 May, p. 326
28. Moenning V, Plagemann PG (1992) The pestiviruses. Adv Virus Res 41: 53–98
29. Nettleton PF (1990) Pestivirus infections in ruminants other than cattle. Rev Sci Tech Off Int Epiz 9: 131–150
30. Orban S, Liess B, Hafez SM, Frey HR, Blindow H, Sasse-Patzer B (1983) Studies on transplacental transmissibility of a bovine virus diarrhoea (BVD) vaccine virus. I. Inoculation of pregnant cows 15 to 90 days before parturition (190th to 265th day of gestation). J Vet Med B 30: 619–634
31. Peters W, Greiser-Wilke I, Moenning V, Liess B (1986) Preliminary serological characterization of bovine viral diarrhoea virus strains using monoclonal antibodies. Vet Microbiol 12: 195–200
32. Potts BJ, Sawyer M, Shekarchi IC, Wismer T, Huddleston D (1989) Peroxidase-labeled primary antibody method for detection of pestivirus contamination in cell cultures. J Virol Methods 26: 119–124
33. Reagan KJ, Goldberg B, Crowell RL (1984) Altered receptor specificity of coxsackievirus B3 after growth in rhabdomyosarcoma cells. J Virol 49: 635–640
34. Rümenapf T, Unger G, Strauss JH, Thiel HJ (1993) Processing of the envelope glycoproteins of pestiviruses. J Virol 67: 3288–3294
35. Sopp P, Hooper LB, Clarke MC, Howard CJ, Brownlie J (1994) Detection of bovine viral diarrhoea virus p80 protein in subpopulations of bovine leukocytes. J Gen Virol 75: 1189–1194

36. Von Boxberg Y, Wütz R, Schwarz U (1990) The use of the biotin-avidin system for labelling, isolation and characterization of neural cell-surface proteins. Eur J Biochem 190: 249–256
37. Weiland E, Stark R, Haas B, Rümenapf T, Meyers G, Thiel HJ (1990) Pestivirus glycoprotein which induces neutralizing antibodies forms part of a disulfide-linked heterodimer. J Virol 64: 3563–3569
38. Xue W, Minocha HC (1993) Identification of the cell surface receptor for bovine viral diarrhoea virus by using anti-idiotypic antibodies. J Gen Virol 74: 73–79

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