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Variation in the Intracellular Polypeptide Profiles from Different Isolates of Bovine Virus Diarrhoea Virus

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With 5 Figures

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Summary

Variation of the intracellular polypeptides induced in calf testis cells by 5 cloned isolates of bovine virus diarrhoea virus (BVDV) was examined. Three of the isolates were cytopathic (NADL, C 2415 and Pe 515 c) and two were non-cytopathic (C 1226 and Pe 515 nc) in these cells. The isolates Pe 515 c and Pe 515 nc were both isolated from an animal with clinical signs of mucosal disease. In cells infected with NADL, 8 virus specific proteins (vp 1 to vp 8) with molecular weights ranging from 120,000 (vp 1) to 23,000 (vp 8) were detected. Isolates C 2415 and Pe 515 c gave a similar array of polypeptides to NADL, but the 3 cytopathic isolates could be distinguished by the variation in the molecular weights of some of the proteins. The non-cytopathic isolates could also be distinguished from each other by this type of molecular variation; however, one feature that characterised these strains, when compared to the cytopathic isolates, was the absence of vp 2. Comparison of the polypeptides induced by Pe 515 c and Pe 515 nc showed that apart from the lack of vp 2 in the Pe 515 nc virus profile, the molecular weights of the other viral proteins were similar. This supports serological evidence that for mucosal disease to occur the pair of cytopathic and non-cytopathic viruses must be closely related. Four of the polypeptides induced by Pe 515 c were shown to be glycoproteins.

Introduction

Bovine virus diarrhoea virus (BVDV) has been classified as a member of the Togaviridae, genus Pestivirus (7). Infection of susceptible cattle during pregnancy results in a number of clinical syndromes affecting the foetus and

newborn calf. Depending on the age of gestation of the foetus at the time of infection the clinical effects can include abortion, teratogenic defects and the birth of persistently infected animals (see reviews, 2, 17). It is these persistently infected animals which later succumb to the usually fatal mucosal disease (12, 14, 22, 23).

Brownlie *et al.* (3) proposed that sequential infection of animals with two forms of the virus, termed non-cytopathic and cytopathic because of their effects on cell cultures, was necessary to precipitate mucosal disease. In their study, only non-cytopathic strains of BVDV were isolated from persistently infected animals but disease occurred following superinfection with a cytopathic isolate. A similar observation has since been made by Bolin *et al.* (1). However the relationship between the cytopathic and non-cytopathic isolates of BVDV was unknown.

Besides variation in cell culture cytopathology, the only other method which showed differences between BVDV isolates was virus neutralization. Fernelius *et al.* (8) found serological variation and proposed the grouping of BVDV strains into serotypes, but later work (5, 10) indicated wider antigenic variation with no evidence of distinct serotypes.

Characterisation of BVDV at the molecular level has so far been limited. The genome of BVDV has been shown to be single stranded RNA of positive polarity (6) although its size has been estimated as either 8.2 kb (19) or 12.5 kb (21). There have also been conflicting reports on the number and size of the virion proteins and virus encoded intracellular polypeptides (4, 15, 18, 20).

This paper reports the analysis of virus induced intracellular polypeptides of five isolates of BVDV. Two non-cytopathic and three cytopathic isolates were selected to give a better understanding of the viruses involved in the mucosal disease syndrome.

Materials and Methods

Viruses and Cell Cultures

Three cytopathic isolates (NADL, C 2415 and Pe 515 c) and two non-cytopathic isolates (Pe 515 nc and C 1226) of BVDV were used. Pe 515 c and Pe 515 nc were isolated from an animal that died during an outbreak of mucosal disease (3). Isolates C 2415 and C 1226, and the American NADL strain were kindly provided by Dr. E. J. Stott (I.A.D.R., Compton, U.K.). Isolate C 2415 came originally from the Central Veterinary Laboratory, Weybridge, U.K. and C 1226 was isolated from a sample of foetal calf serum and has previously been described (16). All the viruses were cloned, the cytopathic isolates by three cycles of plaque purification and the non-cytopathic isolates by three passages at terminal dilution. The viruses were grown in calf testis (CTe) cell cultures, used from passage 4 to 10, and shown to be free from contamination with adventitious BVDV. The cells were grown in Eagle's MEM supplemented with 7.5 per cent heat inactivated foetal calf serum (H-FCS) and 0.25 per cent lactalbumin hydrolysate (LAH), and maintained in BME medium with 2 per cent H-FCS, 0.25 per cent LAH and 30 mM magnesium chloride.

Sera

All antisera were prepared in gnotobiotic calves and were convalescent sera taken 10–12 weeks after intranasal exposure to the individual viruses, as previously described (10). An antiserum produced in a similar way against bovine respiratory syncytial virus served as a control serum.

Labelling of Viral Intracellular Polypeptides

Virus strains were inoculated onto confluent monolayers of CTe cells at a multiplicity of infection of 1 and incubated at 37° C for 40 hours. The medium was replaced with serum-free Earle's BSS and incubated for a further 1 hour. Intracellular viral polypeptides were radioactively labelled by incubating the virus infected cells for 4 hours at 37° C in Earle's BSS containing 50 µ Ci/ml of either L-[^{35}S]-methionine [^{35}]S-met; >800 Ci/mmol, Amersham International plc) or, for glycoprotein labelling, D-[6-^{3}H]-glucosamine hydrochloride (20–40 Ci/mmol, Amersham International plc). The medium was then removed and the cells disrupted, at 4° C, in lysis buffer (0.15 M NaCl, 1 per cent sodium deoxycholate, 1 per cent Triton X-100, 0.1 per cent SDS, 10 mM Tris/HCl pH 7.2, 1 mM phenyl methyl sulphonyl fluoride). After centrifugation at 90,000 × *g* for 30 minutes, the supernatant was removed and stored at −20° C to be used as antigen (Ag) for immune precipitation. Control Ag was prepared from uninfected cultures by the same method.

Immune Precipitation of Viral Polypeptides

For the immune precipitation of viral polypeptides, Ag preparations were preabsorbed with 10 per cent formalinised, heat inactivated *Staph. aureus* (Cowan 1 strain; SaC) in the ratio 4 : 1 (v/v). After incubation at 4° C for 15 minutes the bacteria were removed by centrifugation for 2 minutes in a Beckman Microfuge. Ten µl aliquots of control or anti-BVDV sera, diluted 1 : 4 with phosphate buffered saline, were added to 100 µl samples of preabsorbed Ag. After 1 hour at 20° C, 10 µl 10 per cent SaC were added to each mixture and incubated at 4° C for 15 minutes. The bacteria were pelleted and washed three times with lysis buffer. The adsorbed proteins were recovered by boiling the resuspended bacteria for 2 minutes in 100 µl electrophoresis sample buffer (62.5 mM Tris/HCl pH 6.8; 2 per cent SDS; 2 per cent 2-mercaptoethanol; 10 per cent glycerol containing bromophenol blue as marker dye).

Polyacrylamide Gel Electrophoresis

The polypeptides were separated by electrophoresis on 10 per cent polyacrylamide gels with a 3 per cent stacking gel using the discontinuous buffer system described by LAEMMLI (11). Gels were run at 100 V constant voltage until the marker dye had reached 1 cm above the bottom of the gel. The gels were prepared for fluorography by soaking in En^{3}Hance (New England Nuclear) prior to drying onto filter paper. Fluorography was performed at −70° C with Fuji RX X-ray film.

The apparent molecular weights of the viral polypeptides were determined by comparison with proteins of known molecular weight electrophoresed on the same gel. The protein standards used were the high molecular weight standard mixture (Sigma Chemical Co. Ltd., U.K.) containing myosin (molecular weight 205,000), β-galactosidase (116,000), phosphorylase B (97,400), bovine serum albumin (66,000), ovalbumin (45,000) and carbonic anhydrase (29,000).

Results

Intracellular Polypeptides of BVDV (NADL Strain)

To determine the optimum conditions for radio-labelling the intracellular polypeptides of BVDV, CTe monolayers infected with NADL 40 hours

previously, were labelled with [^{35}S]-met for various lengths of time. The polypeptides detected in these samples by autoradiography after immune precipitation with homologous antiserum are shown in Fig. 1. Eight virus specific polypeptide bands, labelled vp 1 to vp 8, were detected when the labelling period was greater than 4 hours. Both vp 4 and vp 8 were detected as diffuse bands and along with vp 1 and vp 2 were consistently observed in the profiles of the other cytopathic strains tested. In this experiment vp 3 was prominent, but in others, detailed below, this band was difficult to detect. The opposite was found for vp 5 which was seen as a strong band in subsequent profiles. Several other bands were seen, for example between vp 1 and vp 3, but their position and intensity were variable between experi-

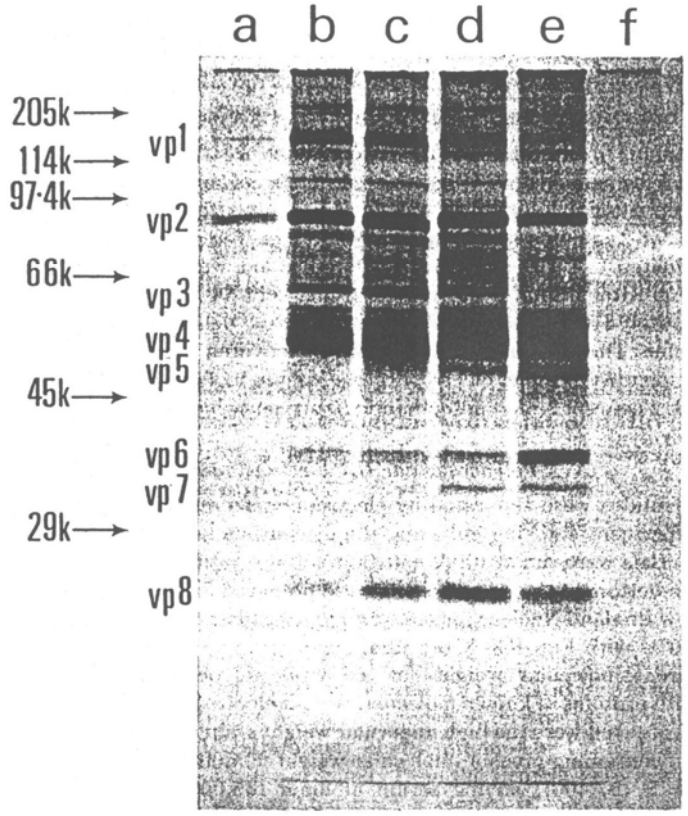

Fig. 1. [^{35}S]-met labelling of NADL intracellular polypeptides. Cell cultures infected with the NADL strain of BVDV, 40 hours p.i., were labelled for 1 hour *(a)*; 2 hours *(b)*; 4 hours *(c)*; 6 hours *(d)*; 24 hours *(e)* and control 40 hours uninfected cells *(f)* labelled for 4 hours. Cell lysates were immune precipitated with NADL antiserum and detected by fluorography after separation through 10 per cent polyacrylamide gels. The BVDV polypeptides are labelled vp 1 to vp 8 and the positions of the protein molecular weight markers are arrowed

ments. The importance of these bands may become clearer when more is known of the replication strategy of the virus. After 24 hours labelling the pattern contained several changes. Both vp 1 and vp 2 were less intense and vp 3 could not be detected, however, vp 5, vp 6 and vp 7 were more prominent. No bands were detected when antigen prepared from uninfected cells was used suggesting that all the bands were of viral origin. For subsequent experiments the labelling period was standardised at 6 hours.

Molecular weights of the polypeptides, estimated by comparison with proteins of known molecular weight, are shown in Table 1 and ranged from 120,000 daltons (vp 1) to 23,000 (vp 8).

Comparison of the Intracellular Polypeptides of Various BVDV Isolates

The polypeptides, precipitated by homologous and heterologous antisera, from the cytopathic NADL and C 2415 strains and by homologous antisera, from non-cytopathic C 1226 and Pe 515 nc are shown in Fig. 2.

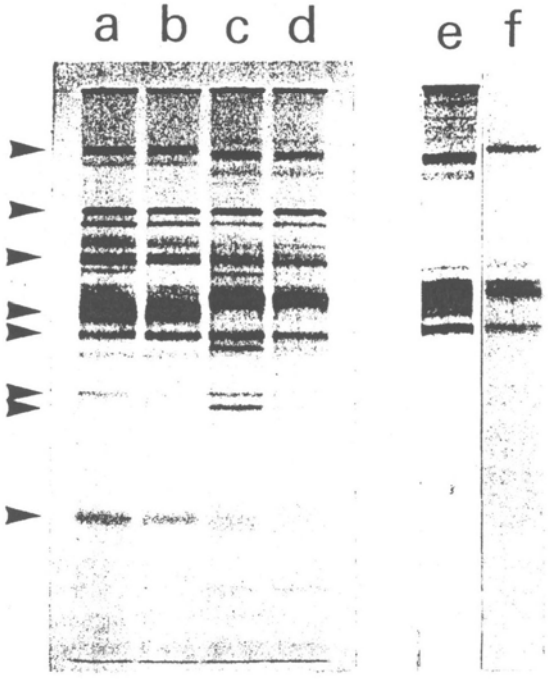

Fig. 2. Comparison of the intracellular polypeptides of two cytopathic and two non-cytopathic BVDV isolates. [^{35}S]-met labelled NADL (*a* and *b*) and C 2415 (*c* and *d*) infected cell lysates were immune precipitated with NADL antiserum (*a* and *d*) and C 2415 antiserum (*b* and *c*). Pe 515 nc *(e)* and C 1226 *(f)* antigen preparations were immune precipitated with homologous antisera. The precipitated polypeptides were separated by electrophoresis and detected by fluorography. Arrows denote the position of the NADL virus polypeptides

Table 1. *Summary of the molecular weights reported for BVDV polypeptides*

BVDV strain	Present paper NADL	Prichett and Zee (18) NADL	Matthaeus (15) NADL and C24V	Coria *et al.* (4) Singer	Purchio *et al.* (19, 20) NADL
vp 1	120,000	93,000–110,000			115,000
vp 2	87,000			75,000	80,000
vp 3	69,000	70,000		66,000	
vp 4	57,000	50,000– 59,000	57,000	54,000	55,000
vp 5	49,000		44,000		45,000
vp 6	37,000		34,000		38,000
vp 7	33,000				
vp 8	23,000	25,000		26,000	

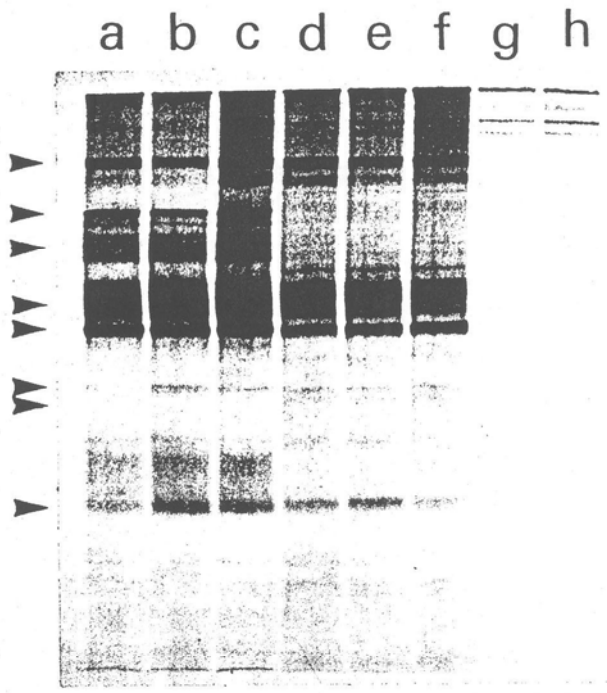

Fig. 3. Comparison of the intracellular polypeptides produced by Pe 515 c and Pe 515 nc. [^{35}S]-met labelled lysates of Pe 515 c infected (*a* to *c*), Pe 515 nc infected (*d* to *f*) and control uninfected (*g* and *h*) cells immune precipitated with antisera against NADL (*a* and *f*), Pe 515 c (*b*, *e* and *g*) and Pe 515 nc (*c*, *d* and *h*). Arrows denote the position of the NADL virus polypeptides

Although the patterns for the two cytopathic isolates were similar, differences in the mobility of vp 1 and vp 4 were seen such that the two isolates could be distinguished. Precipitation of the different Ag preparations by homologous or heterologous antisera showed little difference in the intensity

of most of the polypeptide bands except that the precipitation of vp 6 and vp 7 from C 2415 Ag appeared greater with homologous antiserum.

The two non-cytopathic strains could be distinguished from each other by differences in the mobility of vp 1, and from the cytopathic strains by the striking absence of vp 2 and vp 3. The intensities of the other polypeptide bands, with the exception of vp 1, vp 4 and vp 5, were lower for the non-cytopathic compared to the cytopathic viruses and put into doubt the significance of the failure to detect vp 3.

The comparison of the intracellular polypeptide profiles of the pair of isolates, Pe 515 c and Pe 515 nc, is shown in Fig. 3. Apart from the absence of vp 2 and vp 3 and the lower intensity of other bands in the profile of the non-cytopathic virus, the molecular weights of the corresponding polypeptides of these two viruses were similar. Immune precipitates from Pe 515 nc Ag and antisera to the cytopathic strains Pe 515 c and NADL also lacked vp 2, although these sera were shown to contain antibodies to this polypeptide. Antiserum against Pe 515 nc did, however, contain antibodies to vp 2 and precipitated this polypeptide from preparations of Pe 515 c Ag.

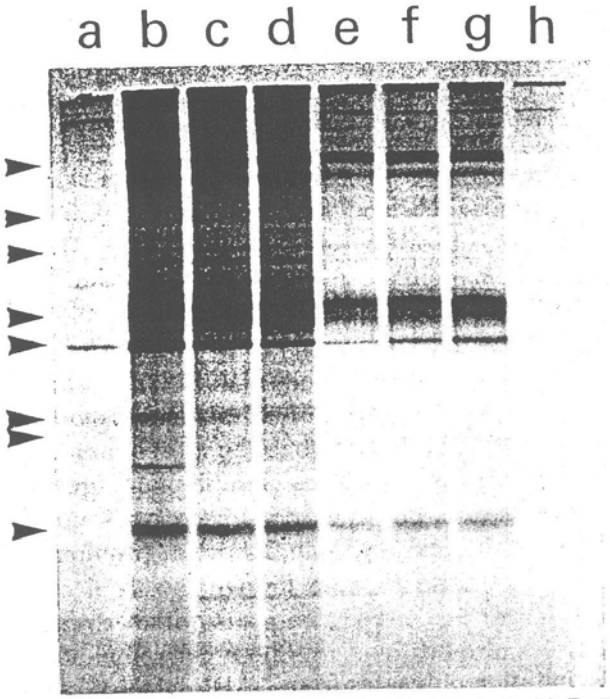

Fig. 4. Detection of intracellular polypeptides in cells infected with Pe 515 nc at different times p.i. Lysates of Pe 515 nc infected cells labelled with [^{35}S]-met at 40 hours p.i. (*a* to *d*) or 4 days p.i. (*e* to *h*) immune precipitated with control (*a* and *h*), Pe 515 c (*b* and *g*), Pe 515 nc (*c* and *f*) and NADL (*d* and *e*) antisera. Arrows denote the position of the NADL virus polypeptides

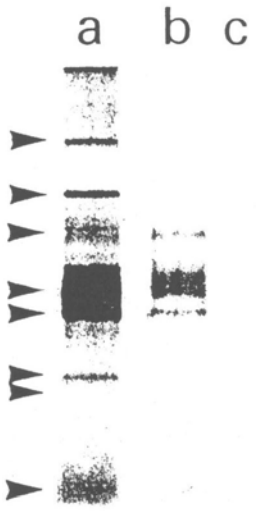

Fig. 5. Analysis of the glycoproteins of BVDV. Pe 515 c polypeptides were labelled with either [^{35}S]-met *(a)* or [^{3}H]-glucosamine *(b)* and control uninfected cells with [^{3}H]-glucosamine *(c)*. These Ag preparations were immune precipitated with Pe 515 c antiserum and the polypeptides separated by electrophoresis. Arrows denote the position of the NADL polypeptides

To ensure that vp 2 was not produced later than 40 hours p.i., Ag was prepared from Pe 515 nc infected cells 4 days p.i. but the polypeptide was not detected with antisera to either cytopathic or non-cytopathic strains (Fig. 4).

Detection of BVDV Glycoproteins

After labelling Pe 515 c infected cells, 40 hours p.i., with [^{3}H]-glucosamine four labelled polypeptides were immune precipitated (Fig. 5). These polypeptides were found to correspond to vp 3, vp 4, vp 5 and vp 8 when compared with the [^{35}S]-met labelled viral proteins.

Discussion

Immune precipitation of lysates from NADL infected cell cultures with a convalescent serum detected eight virus specific polypeptides (vp 1–vp 8) having molecular weights from 23,000 to 120,000 daltons. The protein patterns of two other cytopathic BVDV strains, C 2415 and Pe 515 c were similar, but minor changes in the molecular weights of some proteins

characterised each strain. Four of the polypeptides (vp 3, vp 4, vp 5 and vp 8) precipitated from Pe 515 c infected cells were shown to be glycosylated. The absence of vp 2 in the profiles of the two non-cytopathic strains tested distinguished them from the cytopathic viruses.

Although more viral polypeptides were detected in our studies, the size range is in agreement with other published data (Table 1). These reports, with the exception of the paper by PURCHIO *et al.* (20), dealt with the structural components of BVDV. A larger number of polypeptides would be expected in infected cells because of the possibility of non-structural components and precursor virion proteins. PURCHIO *et al.* (20) analysed the intra-cellular polypeptides from BVDV infected cells with similar techniques but found only 5 viral proteins (consistent with vp 1, vp 2, vp 4, vp 5 and vp 6 reported here) all of which appeared to be associated with mature virions. This discrepancy may be due to differences in antisera used for immune precipitation. The sera raised in gnotobiotic animals used in this study probably gave better precipitation of all BVDV polypeptides because of the higher specific activity of BVDV antibodies.

Serological evidence has suggested that the production of mucosal disease requires the pair of cytopathic and non-cytopathic BVDV strains to be antigenically closely related (10). This could explain the unsuccessful attempt to produce the disease experimentally by the superinfection of a viraemic animal with cytopathic virus (9). Our observed variation in the protein profiles of the various strains suggests that this type of analysis could prove useful in studying pairs of BVDV isolates and their relationship to disease. The finding that the polypeptide profiles of Pe 515 c and Pe 515 nc, except for vp 2 and vp 3, showed the closest similarity of any of the strains tested may prove significant in this context.

By tryptic peptide mapping, BVDV proteins vp 1 and vp 2 were found to be structurally related (20), and suggested some post-translational processing of vp 1. This would explain the precipitation of vp 2 from cytopathic virus Ag preparations by antiserum to Pe 515 nc. Comparison of the growth of cytopathic and non-cytopathic isolates in cell cultures has shown that while the initial replication kinetics of both viruses were similar (16) the maximum titres reached were 2 $\log_{10}$ lower with non-cytopathic viruses (13, 16). Therefore, the lack of cytopathology shown by non-cytopathic strains may be due to their slower overall multiplication rate. It is tempting to speculate that the reason for this might be the lack or lower rate of conversion of vp 1 to vp 2, indicating a difference in the primary structure of vp 1, derived from the different types of virus, at the cleavage site.

Work is in progress to characterise additional pairs of cytopathic and non-cytopathic BVDV isolated from animals in other outbreaks of mucosal disease to give a better understanding of the relationship between the pairs of viruses involved in the pathogenesis of disease.

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