Cytopathic and non-cytopathic biotypes of border disease virus induce polypeptides of different molecular weight with common antigenic determinants

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Ten monoclonal antibodies have been raised against lysates of cells infected with cytopathic border disease virus (BDV). These antibodies all recognize non-cytopathic BDV and react with a number of different strains of bovine viral diarrhoea virus (BVDV). Studies with radiolabelled cell lysates show that all the antibodies precipitate two polypeptides of apparent Mr 80K and 130K from cells infected with cytopathic virus and a single polypeptide of apparent Mr 120K from cells infected with non-cytopathic virus. Two of the monoclonal antibodies react on immunoblots and show the same pattern of reactivity indicating that these three polypeptides are antigenically related.

Border disease virus (BDV) is a pestivirus which causes widespread congenital disease in sheep (Westaway et al., 1985). It consists of two biotypes, a cytopathic (CP) and a non-cytopathic (NCP) virus which produce different pathologies in pregnant sheep. Infection of pregnant ewes with NCP virus at 52 days gestation results in the birth of persistently infected lambs with neonatal abnormalities whereas infection with the CP virus at the same stage in gestation does not cause identifiable disease (Nettleton et al., 1987). Persistently infected lambs can excrete NCP virus for long periods of time but may succumb at any age to spontaneous or experimentally induced 'mucosal disease' when CP virus is readily recoverable from the animal (Gardiner et al., 1983).

BDV is antigenically related to the bovine pestivirus bovine viral diarrhoea virus (BVDV) (Osburn et al., 1973). Although there is evidence that BDV is serologically distinct from BVDV (Nettleton, 1987), cross-species infections can occur and the viruses have not been clearly differentiated (Edwards et al., 1988). The genome of BVDV has been cloned and sequenced (Renard et al., 1985; Collett et al., 1988a) and studies using sera raised against fusion proteins containing putative virus protein sequences have identified protein products encoded by the virus genome (Collett et al., 1988b). In addition, radiolabelled polypeptides induced in infected cells have been identified (Donis & Dubovi, 1987a, b) and monoclonal antibodies against virus-induced proteins have been described (Peters et al., 1986; Donis et al., 1988; Bolin et al., 1988). In contrast, little is known about BDV polypeptides. We have chosen to investigate the BDV polypeptides by raising monoclonal antibodies to CP BDV-infected cell lysates. In this paper we show that these antibodies recognize both biotypes but they react with two antigenically related polypeptides of apparent Mr 80K and 130K induced in CP virus-infected cells, whereas they recognize a single polypeptide of 120K from NCP virus-infected cells.

The BDV stocks used were the Moredun strain CP and NCP virus. These viruses were derived by cloning from the JIB brain pool virus stock (Barlow, 1972). This stock is cytopathic in foetal lamb kidney (FLK) cells (Vantsis et al., 1976) but in vivo and in vitro studies have shown that it contains CP and NCP biotypes. Both CP and NCP viruses were propagated in FLK or foetal lamb muscle (FLM) cells grown in roller bottles (Jencons). Briefly, FLK or FLM cells were seeded into roller bottles and grown to confluence in Iscove's modified Dulbecco's medium (IMDM; Gibco) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 10% foetal bovine serum (FBS; Flow Laboratories). All batches of FBS used were determined to be free of BVDV and anti-BVDV antibody by three passages in tissue culture on virus-permissive cells and by neutralization and indirect immunofluorescence tests. The confluent monolayers were washed three times with Hanks' balanced salt solution (HBSS) and inoculated with either CP or NCP virus at a multiplicity of infection of <0·1 infectious units per cell. The inoculum was removed after 2 h and replaced with IMDM plus 2% FCS as maintenance medium. Supernatants containing virus were harvested after 4 to 5 days.
For immunization of mice, cells were infected with CP virus as described above and, after the removal of the supernatant virus, harvested from flasks using glass beads. After centrifugation at 400 g the cells were washed twice with phosphate-buffered saline (PBS). The pellet was resuspended in a small volume of PBS and the cells were lysed by three cycles of rapid freezing and thawing in liquid nitrogen. The resulting lysate was clarified by centrifugation at 3000 g then stored at −80 °C. Ten to 12 week old female BALB/c mice were immunized with a primary intraperitoneal (i.p.) injection of 0.2 ml of lysate emulsified 50% (v/v) with Freund’s complete adjuvant (Difco Laboratories). This was followed 3 weeks later by a secondary i.p. injection of lysate emulsified 50% (v/v) with Freund’s incomplete adjuvant. The mice were rested for a period of 6 to 8 months before receiving a final intravenous boost of lysate in PBS 3 days prior to fusion. Spleen cells from immunized mice were fused with NS0 cells by the method described by Fazekas de St Groth & Scheidegger (1980). Culture supernatants from wells containing hybrids were screened for antibody 10 to 14 days post-fusion by an indirect fluorescent antibody test on infected cell monolayers. Indirect immunofluorescence on hybrid supernatants was performed using affinity-purified sheep anti-mouse IgG antiserum labelled with fluorescein isothiocyanate as a second step reagent.

Hybrids secreting anti-BDV antibody were cloned three times by limiting dilution. Ascitic fluids were produced by injecting pristane-primed mice i.p. with 5 × 10⁶ cloned hybridoma cells (Brodeur et al., 1984). The monoclonal antibodies were isotyped using specific antisera to mouse immunoglobulins (The Binding Site Ltd). Purified Ig was prepared by affinity chromatography on Protein A-Sepharose (Sigma).

For immunoprecipitation studies FLM cells were grown to 75% confluence in 50 mm tissue culture dishes in Dulbecco’s modified medium (DMEM) containing 10% FBS and infected at a multiplicity of infection of 0.5 to 1 infectious units per cell. Virus was adsorbed to the cells for 2 h at 37 °C and the infected or mock-infected cells were incubated for a further 22 h before labelling for 4 h with 100 μCi/ml [35S]methionine (Amersham) in methionine-free medium (Eagle’s minimal essential medium; Gibco). The cells were harvested by removing the medium, washing the monolayers twice with HBSS and adding 1 ml lysis buffer (20 mM-Tris–HCl pH 8.0, 150 mM-NaCl, 0.5% sodium deoxycholate, 0.5%, NP40, 0.2 mM-PMSF) (Showalter et al., 1981). The plates were frozen at −70 °C, thawed and the cells were scraped into the buffer. The lysate was further disrupted by ultrasonic treatment and clarified by centrifugation at 10000 g for 10 min. The amount of radioactivity incorporated into protein was determined by TCA precipitation.

Immunoprecipitation was carried out by a solid phase method as follows. ELISA plates (Dynatech) were coated overnight with 50 μl purified immunoglobulin (150 μg/ml), washed three times in 0.1% Tween 20 in PBS (PBS/Tween) and incubated without blocking overnight at 4 °C with 2 × 10⁵ TCA-precipitable c.p.m. The wells were further washed three times with PBS/Tween and eluted into 50 μl elution buffer (125 mM-Tris–HCl pH 6–8, 2% w/v SDS, 5% w/v 2-mercaptoethanol, 0.04% w/v bromophenol blue) by heating in a boiling water bath for 2 min. The samples were fractionated on 5 to 15% gradient SDS-polyacrylamide gels (Laemmli, 1970), which were then impregnated with En3Hance (New England Nuclear) and fluorographed against Kodak XS-1 film.

For immunoblotting studies, 2 × 10⁶ cells were infected as described for immunoprecipitation and harvested 28 h post-infection directly into 0.5 ml SDS elution buffer. Fifteen μl aliquots of cell lysate were separated on 5 to 15% SDS–polyacrylamide gels and transferred to nitrocellulose using an Anco semi-dry blotter according to the manufacturer’s directions. The membrane was blocked with PBS containing 5% (w/v) dried milk and 0.05% Tween 20 and incubated overnight at room temperature on a roller in monoclonal antibody supernatant. The membrane was washed five times for at least 5 min with 1% dried milk in PBS containing 0.05% Tween 20 (wash buffer), incubated for 1 h with a 500-fold dilution of biotinylated goat anti-mouse Ig (Sigma), washed as before, incubated for 1 h with extravidin–alkaline phosphatase (Sigma) diluted 200-fold, washed and developed using the Bio-Rad alkaline phosphatase substrate kit.

Ten monoclonal antibodies, designated VPM20, VPM21, VPM22, VPM23, VPM24, VPM25, VPM26, VPM27, VPM28 and VPM49, were raised against CP BDV-infected cell lysates. VPM20, 23, 25, 26, 27, 28 and 49 are IgG2a isotype, VPM21 and 24 are IgG1 and VPM22 is IgG2b. The cross-reactivity of the antibodies against various pestivirus isolates was investigated by indirect immunofluorescence on infected cell cultures. They all reacted with the CP BDV-infected cell cultures. VPM20, 23, 25, 26, 27, 28 and 49 are IgG2a isotype, VPM21 and 24 are IgG1 and VPM22 is IgG2b. The cross-reactivity of the antibodies against various pestivirus isolates was investigated by indirect immunofluorescence on infected cell cultures. They all reacted with the CP BDV-infected cell cultures. VPM20, 23, 25, 26, 27, 28 and 49 are IgG2a isotype, VPM21 and 24 are IgG1 and VPM22 is IgG2b.
the antibodies fail to react with one or more of the virus strains tested (S. Edwards, personal communication).

Fig. 1 shows immunofluorescence staining of FLM cells infected with Moreduin CP and NCP BDV. A similar staining pattern is seen with both biotypes. Neutralization tests against the immunizing virus showed that none of the antibodies was capable of neutralizing infectivity. The intensity of the staining was stronger with the pool than with individual antibodies, further suggesting that a number of different epitopes are recognized.

Immunoprecipitations were carried out in order to identify the antigens recognized by the monoclonal antibodies. Initial studies involved incubating [$^{35}$S]methionine-labelled CP- and NCP-infected and mock-infected FLM cell lysates with ascitic fluid and precipitating antigen-antibody complexes with sheep anti-mouse Ig coupled to Affigel 10, or with Protein A–Sepharose (Sigma) or Pansorbin (Calbiochem). These experiments suggested that the antibodies were reacting with two polypeptides of apparent $M_r$ 80K and 130K from CP virus-infected cells and one polypeptide of apparent $M_r$ 120K from NCP virus-infected cells. However, a number of unrelated control antibodies including an IgG2b ascites purchased from Sigma also precipitated these polypeptides to varying degrees, raising the possibility that the precipitation was non-specific. The situation could not be resolved by stringent washing in buffers containing 0-1% SDS. Similar precipitation of the BVDV 80K polypeptide has been observed by others (Donis et al., 1988) and it is likely that these proteins have a high affinity for sheep Ig, Protein A or agarose beads. Identification of the antigens recognized by the monoclonal antibodies was resolved by coating purified monoclonal antibodies directly onto ELISA plates and adding the labelled lysate to the plate, thus avoiding a precipitation step. The results of such an experiment are shown in Fig. 2. All the anti-BDV antibodies react with an 80K and a 130K polypeptide induced in CP BDV-infected cells and a 120K polypeptide induced in NCP-infected cells. No infected cell polypeptides react with VPM16, an anti-sheep major histocompatibility complex class II monoclonal antibody used as a negative control.

The coprecipitation of the 80K and the 130K polypeptides from CP BDV-infected cells might occur because the proteins are structurally associated in the infected cell and are not dissociated by the washing procedures or it might indicate that they have a common antigenic determinant. To investigate this, immunoblotting studies were carried out. Two of the monoclonal antibodies, VPM22 and VPM24, reacted with protein transferred to nitrocellulose and a blot with VPM22 is shown in Fig. 3. VPM22 shows the same reactivity on immunoblots as in immunoprecipitation indicating that the 80K and 130K polypeptides have a common antigenic determinant.

The monoclonal antibodies described here are the first characterized against BDV and provide important information on the polypeptides induced in infected cells by this virus. To date, little data concerning the BDV polypeptide composition has been available and it has been assumed to be similar to BVDV. Early peptide mapping studies on BVDV showed that the 80K polypeptide present in CP BVDV-infected cells was related to the 120K CP BVDV-induced polypeptide (Purchio et al., 1984). More recent studies using anti-peptide antisera have confirmed that the BVDV 80K polypeptide is encoded within the 120K polypeptide (Collett et al., 1988b) and is presumably derived by proteolytic cleavage. The results presented here show that CP BDV induces a similar pair of polypeptides and analogy with BVDV strongly suggests that they are BDV-encoded products.

NCP strains of BVDV differ from CP strains in that
they fail to induce synthesis of a polypeptide comparable in $M_r$ to the cytopathic 80K protein (Pocock et al., 1987; Donis & Dubovi, 1987c). They do, however, induce synthesis of a polypeptide of approximately 120K comparable in size to the higher $M_r$ CP virus protein. The results presented here show that NCP BDV also fails to induce an 80K equivalent but induces synthesis of a 120K polypeptide. The immunoblotting experiments indicate that this protein is related to the CP 130K and 80K polypeptides. Interestingly, there is a distinct size difference between the CP and NCP higher $M_r$ proteins. The significance of this is not clear because although both viruses are derived from the same brain pool (IIB) and are assumed to be a homologous pair, we cannot exclude the possibility that other viruses may have been present in the original isolate.

The monoclonal antibodies described here do not distinguish the CP and NCP biotypes of BDV and also fail to discriminate between BDV and BVDV. Serological studies have indicated that BDV strains are distinct from BVDV strains although at least one sheep isolate has been found which is more closely related to BVDV than to the BDV Moredun strain (Vantsis et al., 1980; Nettleton, 1987). In a study of 74 anti-pestivirus monoclonal antibodies none was BDV-specific, although eight hog cholera virus-specific and three BVDV-specific antibodies were detected (Cay et al., 1989). Thus the relationship between BVD and BD viruses remains to be resolved.

A number of other anti-pestivirus monoclonal antibodies have been reported (Peters et al., 1986; Wenswoort et al., 1986; Bolin et al., 1988; Donis et al., 1988). Only monoclonal antibodies to BVDV have been characterized in detail and the majority react with the 56K to 58K glycoprotein. In contrast, all our antibodies react with the 130K and 80K (CP) and 120K (NCP) BDV-induced polypeptides. It is likely that this arises because the mice were immunized with infected cell lysates rather than purified virus. Although the BDV 120K and 80K polypeptides are present in some purified virus preparations (Purchio et al., 1984) they probably represent a much smaller percentage of virus protein than is found in infected cells. Alternatively, the immunization regime may be important for the antigenicity of these polypeptides.

Peters et al. (1986) have described a CP BVDV-
specific monoclonal antibody that recognizes a 79K polypeptide and other CP BVDV-specific monoclonal antibodies of 125K/80K specificity are reported in a review by Collett et al. (1989). None of our anti-BDV monoclonal antibodies distinguish CP and NCP biotypes and there may be a limited number of epitopes unique to either of these proteins. However, the success of the immunization strategy employed to raise these antibodies together with the obvious importance of these proteins in distinguishing between CP and NCP virus suggests that the approach may be extremely fruitful.

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References


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