Journal of General Virology (1992), 73, 2591–2600. *Printed in Great Britain*

Synthesis and processing of the haemagglutinin–esterase glycoprotein of bovine coronavirus encoded in the E3 region of adenovirus

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The haemagglutinin–esterase gene (HE) of bovine coronavirus (BCV) encodes a major viral membrane glycoprotein that elicits BCV-neutralizing antibodies. The BCV HE gene was cloned into a human adenovirus serotype 5 (Ad5) transfer vector in place of early transcription region 3, and a helper-independent recombinant virus was constructed by rescue of the transcription unit by homologous *in vivo* recombination between the vector and Ad5 genomic DNA. The BCV HE polypeptide expressed by this recombinant Ad was characterized *in vivo* and *in vitro*. A 65K polypeptide was identified using an anti-BCV antibody in both human (293) and bovine (MDBK) cells infected with the recombinant Ad. In the absence of a reducing agent, migration of the 65K polypeptide was shifted to 130K, indicating that the recombinant HE polypeptide existed in a dimeric form. The HE polypeptide was glycosylated, as demonstrated by labelling with [^{3}H]glucosamine, and was immunoreactive with three distinct groups of conformation-specific anti-HE monoclonal antibodies (MAbs). Cells infected with recombinant Ad expressing BCV HE exhibited both haemadsorption activity and acetylesterase activity. In addition, the anti-HE group A MAbs HC10-5 and KD9-40 inhibited both the haemadsorption activity and esterase activity of the recombinant HE polypeptide, suggesting that the antigenic domain responsible for BCV neutralization may overlap (or is closely associated with) the domain(s) responsible for haemagglutination and/or acetylesterase activities. When mice were inoculated intraperitoneally with live recombinant Ad, a significant level of BCV-neutralizing HE-specific antibody was induced. These results indicate that the recombinant Ad replicates and directs the synthesis of the BCV HE polypeptide *in vivo*.

Introduction

Coronaviruses contain a large, positive-sense ssRNA of approximately 30 kb in length that is associated with nucleocapsid (N) protein (55K) in a helical ribonucleoprotein complex (MacNaughton *et al.*, 1978). In addition to the N protein, two membrane glycoproteins are found in virions: the spike (S) protein (180K) and the integral membrane (M) protein (23K). The S glycoprotein forms surface peplomers and functions in virus attachment (Collins *et al.*, 1982) and cell fusion (Sturman *et al.*, 1985; de Groots *et al.*, 1989; Yoo *et al.*, 1991), whereas the M protein determines the site of virus maturation (Tooze *et al.*, 1984; Rottier & Rose, 1987). A third membrane glycoprotein, haemagglutinin–esterase (HE; 65K), is found only in certain species of coronaviruses, such as bovine coronavirus (BCV) (King & Brian, 1982; King *et al.*, 1985; Deregt *et al.*, 1987), haemagglutinating encephalomyelitis virus (Callebaut & Pensaert, 1980) and human coronavirus OC43 (Hogue & Brian, 1986). In strains of mouse hepatitis virus (MHV), the HE gene is present but is expressed in only a few isolates (Makino & Lai, 1989; Shieh *et al.*, 1989). It appears that the HE gene in MHV-JHM is a pseudogene which is not essential for virus replication (Yokomori *et al.*, 1991). Moreover, the HE gene does not exist at all in avian infectious bronchitis virus (Stern & Sefton, 1982; Boursnell *et al.*, 1987) or porcine transmissible gastroenteritis virus (Garwes & Reynolds, 1981). In contrast, the HE protein of BCV is a major membrane-associated glycoprotein and is essential for virus replication (Vlasak *et al.*, 1988*b*). The BCV HE has been reported to exhibit significant sequence homology with the haemagglutinin of type C human influenza virus (Nakada *et al.*, 1984; Luytjes *et al.*, 1988; Parker *et al.*, 1989, 1990*b*), having known haemagglutination and acetylesterase functions

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(Vlasak *et al.*, 1988*a*, *b*). Furthermore, previous studies have demonstrated that monoclonal antibodies (MAbs) directed against the HE glycoprotein efficiently neutralize BCV infectivity *in vitro* (Deregt & Babiuk, 1987) and are capable of protecting the intestinal epithelia of cattle from virus infection (Deregt *et al.*, 1989). These reports indicate a significant role for this protein in the initiation of BCV infection.

For several years the development of live modified virus vectors has attracted considerable attention for use as an effective and potentially inexpensive way of controlling virus infections. Studies using vaccinia virus have demonstrated the usefulness of infectious virus vectors in inducing protective immune responses in animals (for a review see Piccini & Paoletti, 1988). Unattenuated live adenovirus (Ad) has been used for oral immunization against acute respiratory disease in military recruits for over 20 years, and since it has been proven to be safe and efficacious (Couch *et al.*, 1963; Top, 1975) it is an attractive live vector for antigen delivery. Recently, Ad5 has been developed as a eukaryotic expression vector, and three regions in the Ad genome have mainly been utilized for the insertion of foreign DNA: early transcription region 1 (E1), E3 and the region between the start of E4 and the right inverted terminal repeat (for a review see Berkner, 1988; Graham, 1990; Graham & Prevec, 1991*b*). Deletion of E1 results in a replication-defective virus that grows only in 293 cells, which provide E1 functions in trans (Graham *et al.*, 1977; Aiello, 1979; Berk *et al.*, 1979). In contrast, E3 is non-essential and can be deleted without affecting the ability of the virus to replicate in cultured cells (Berkner & Sharp, 1983). The third region has also been used for high-level expression of inserted genes (Saito *et al.*, 1985; Mason *et al.*, 1990). We constructed an infectious Ad vector carrying the BCV HE gene in the E3 locus of the Ad5 genome, and in this report we describe the biochemical and biological properties of the recombinant HE polypeptide synthesized by this vector, and discuss the potential use of live Ad vectors in animals.

Methods

Cells, viruses and antibodies. BCV (Quebec isolate) was grown in Mardin-Darby bovine kidney (MDBK) cells in MEM supplemented with 10% foetal bovine serum (FBS) (Gibco). Wild-type human Ad5 and recombinant Ad (AdBcHE) were titrated and propagated in 293 cells (Graham *et al.*, 1977) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS plus additional amino acids and vitamins (Flow Laboratories). For animal infections, recombinant Ad was pelleted through a 30% sucrose cushion and purified by subsequent centrifugation in a discontinuous caesium chloride gradient with a density of 1·2 to 1·4 g/ml (Green & Wold, 1979). Virus bands were collected and the buffer was exchanged through a Sephadex G-25M PD-10 column (Pharmacia). The virus inoculum was titrated on 293 cells and resuspended in 1 mM-EDTA, 100 mM-Tris–HCl pH 8·0. Polyclonal rabbit anti-BCV antiserum and mouse ascites fluid of MAbs HC10-5, BD9-8C, KD9-40 and KC4-3 were prepared as described previously (Deregt & Babiuk, 1987).

DNA cloning. Enzymes were purchased from Pharmacia and Boehringer Mannheim. DNA was manipulated according to standard procedures (Sambrook *et al.*, 1989). The entire open reading frame of the BCV HE gene was obtained from pCVE3 (Parker *et al.*, 1989) by digestion with *Bam*HI, and subcloned into the *Bam*HI site of pSV2X3 (Prevec *et al.*, 1990). Plasmids containing the HE gene in the desired orientation downstream of the simian virus 40 (SV40) promoter were identified by restriction endonuclease analysis. An expression cassette consisting of the SV40 promoter and a polyadenylation signal flanking the HE gene was isolated from pSV2X3 by *Xba*I digestion, and the resulting fragment was subcloned into the unique *Xba*I site of the Ad transfer vector pFGdX1 (Haj-Ahmad & Graham, 1986). The recombinant transfer vector pFGBCE3, for cotransfection, was prepared by CsCl gradient purification as described (Sambrook *et al.*, 1989).

DNA transfection, screening of recombinant virus and plaque assays. Monolayers of 293 cells were cotransfected with 10 μg of the transfer vector plasmid pFGBCE3 and 5 μg of *Eco*RI-digested wild-type Ad5 DNA using the calcium phosphate precipitation technique as described previously (Graham & van der Eb, 1973). After transfection, cells were overlaid with 1% agarose and incubated at 37 °C for 5 to 8 days until plaques developed. Well-isolated plaques were picked by punching out agar plugs with a sterile Pasteur pipette and resuspended in 1 ml of PBS containing 10% glycerol. The isolated virus plaques were recultured in 293 cells, and when a complete c.p.e. developed cells were collected and digested with pronase (0·5 mg/ml plus 0·5% SDS) for 3 h. DNA from the cell lysates was extracted with phenol/chloroform and precipitated with ethanol. The crude DNA was redissolved in 0·1 × SSC and digested with *Hin*dIII for 4 h, and then analysed on a 1% agarose gel (Graham & Prevec, 1991*a*). Recombinant Ads containing the correct restriction pattern were purified further by two additional plaque assays in 293 cells and amplified for virus stock. The stock virus was titrated and stored in 10% glycerol at −80 °C.

Radiolabelling and preparation of cellular extracts. Confluent monolayers of 293 cells or MDBK cells in 60 mm dishes were infected with BCV or with Ad at a multiplicity of 10. MDBK cells infected with BCV were labelled between 18 and 24 h post-infection and the Ad-infected MDBK cells were labelled for 6 h at 12 h post-infection. In each case, the cells were pre-incubated for 30 min in medium deficient of methionine prior to radiolabelling. Cells were labelled with 50 μCi/ml [^{35}S]methionine (Amersham, 1000 Ci/mmol). For glycosylation studies, virus-infected cells were labelled with 50 μCi/ml D-[6-^{3}H]glucosamine hydrochloride (Amersham, 25·4 Ci/mmol) in glucose-free Hanks' balanced salt solution (Gibco). Cells were washed once with PBS and immediately harvested by centrifugation at 2000 r.p.m. for 10 min, and then lysed with 0·5% Triton X-100, 150 mM-NaCl, 50 mM-Tris–HCl pH 7·5 by incubating for 10 min on ice. The cell lysates were centrifuged at full speed in a microcentrifuge, and cytoplasmic fractions were collected and used for immunoprecipitation.

Immunoprecipitation and SDS–PAGE. Antibodies were pre-incubated for 2 h at room temperature with cell lysates prepared from mock-infected cells. The pre-incubated antibody was then added to the radioactively labelled samples prepared from virus-infected cells, and the mixtures were further incubated for 2 h at room temperature. Protein A–Sepharose beads (10 mg; Pharmacia) were added to the mixture, and the mixtures were incubated overnight at 4 °C with continuous shaking in RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 150 mM-NaCl, 50 mM-Tris–HCl pH 7·5, 10 mM-EDTA, 0·5% SDS). Immune complexes bound to Sepharose beads were washed

three times with RIPA buffer and dissociated by boiling for 5 min in 10% SDS, 25% glycerol, 0·02% bromophenol blue, 10 mM-Tris–HCl pH 6·8 in the presence or absence of 10% 2-mercaptoethanol. Polypeptides were resolved on 10% SDS–polyacrylamide gels at 15 V/cm^2. The gels were fixed in 40% methanol containing 10% acetic acid, treated with Amplify (Amersham) and autoradiographed at −80 °C.

Acetylesterase assay. Acetylesterase activity was determined by measuring the amount of acetic acid released from 9-*O*-acetylsialic acid after incubation of virus-infected cells with substrates as described (Vlasak *et al.*, 1988*a*). MDBK cells (5×10^6) were resuspended in 250 µl of PBS and incubated at room temperature with 50 µl of bovine submaxillary mucin (25 mg/ml; Boehringer Mannheim). At different times, the amount of acetic acid released from the mucin was measured by u.v. absorption using a commercially available acetic acid determination kit (Boehringer Mannheim). Antibody-mediated inhibition of esterase activity was determined by pre-incubation of the samples with an appropriate antibody for 1 h prior to the addition of substrate.

Haemadsorption assay. BALB/c mouse erythrocytes were washed twice with Alsever's solution (100 mM-glucose, 20 mM-sodium citrate, 70 mM-NaCl, 2 mM-citric acid) and resuspended in Alsever's solution to 20% erythrocytes. MDBK cells at 18 h post-infection were rinsed twice with PBS and incubated with 0·5% mouse erythrocytes for 10 min at room temperature. For antibody inhibition assays, cells were pre-incubated for 1 h with MAbs diluted 1:100 in PBS. Cell monolayers were washed twice with PBS and incubated for 10 min with the mouse erythrocytes at room temperature. Binding of erythrocytes to virus-infected cells was monitored by microscopic examination. For quantification of haemadsorption activities, mouse erythrocytes were labelled with sodium [^{51}Cr]chromate (Amersham, 200 mCi/mg) and the radiolabelled erythrocytes were added to virus-infected cells. After a 10 min incubation at room temperature, monolayers were washed five times with PBS to remove unbound erythrocytes. Cell-bound erythrocytes were lysed by addition of lysis buffer (Triton X-100, 150 mM-NaCl, 10 mM-Tris–HCl pH 7·6), and the radioactivity of the cell lysates was measured with a gamma radiation counter (Beckman Model G 5500).

Immunofluorescence assay. To determine the distribution of antigen on the cell surface, MDBK cells were grown on a Lab-Tek chamber slide (Miles Laboratories) and infected with AdBcHE at a multiplicity of 5 p.f.u./cell. At 18 h post-infection, unfixed cells were washed with cold PBS and incubated for 1 h with a 1:300 dilution of a pool of anti-HE MAbs. Cells were then washed with PBS and reacted with a 1:100 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Boehringer Mannheim) for 1 h. Cells were washed again with PBS and examined for fluorescence (Zeiss microscope Model IM35).

Animal immunization. Three-week-old Swiss mice were obtained from the Animal Breeding Laboratory, Western College of Veterinary Medicine, University of Saskatchewan, Canada. Mice were inoculated intraperitoneally with 10^8 p.f.u. of recombinant Ad, AdBcHE, in a 200 µl volume. Blood samples were periodically taken from the tail veins of the immunized mice, and sera were separated and stored at −20 °C for antibody determination.

ELISA. Serum antibody responses against BCV were analysed by ELISA. Approximately 50 ng of virions in coating buffer (50 mM-$NaHCO_3/Na_2CO_3$ pH 9·6) were coated onto each well of Immulon 2 Microtitre plates (Dynatech Laboratories) at 4 °C overnight. The plates were then washed four times with distilled water, and incubated with 3% heat-inactivated horse serum (JR Scientific) in 10 mM-PBS for 1 h at room temperature. The plates were washed with washing buffer (10 mM-PBS pH, 7·2, 0·05% Tween 20), and the mouse serum, serially diluted in dilution buffer (10 mM-PBS pH 7·2, 0·85% NaCl, 0·05% Tween 20), was added and incubated for 2 h at room temperature. After removing unbound antibody by washing the plates three times with washing buffer, a 1:6000 dilution of biotin-conjugated rabbit anti-mouse IgG (Zymed Laboratories) containing 1% horse serum was added, followed by a 1 h incubation at room temperature. The plates were then incubated with a 1:2000 dilution of streptavidin–horseradish peroxidase conjugate (Zymed Laboratories) for 1 h at room temperature. After washing of the plates with washing buffer, 0·15 mg/ml of 2,2′-azino-di-[3-ethyl-benzthiazoline sulphonate(6)] (ABTS; Boehringer Mannhein) in 50 mM-citric acid pH 4·0 containing 0·01% hydrogen peroxide was added for colour development. The enzyme reaction was terminated by the addition of an equal volume of 10% SDS. The absorbance at 405 nm (A_{405}) with a reference wavelength at 490 nm was determined using a microplate reader (Bio-Rad, Model 3550). The amount of HE antigen in virus-infected cells was determined by a sandwich ELISA. Immulon 2, 96-well microtitre plates were sensitized with rabbit anti-mouse IgG (Zymed Laboratories) diluted to 1:800 in coating buffer at 4 °C overnight. The plates were washed with distilled water four times and incubated for 1 h with a pool of HE-specific MAbs diluted to 1:10000 in dilution buffer. After washing with washing buffer four times, a 1:100 dilution of cytoplasmic fractions prepared from virus-infected cells was added, followed by incubation for 1 h. The plates were washed again and incubated with a 1:80 dilution of rabbit anti-BCV antibody conjugated with horseradish peroxidase. The colour was developed by adding ABTS, and the absorbance was measured.

Serum neutralizing antibody assays. Neutralizing antibody responses were determined by incubating twofold dilutions of antisera with 50 $TCID_{50}$ of BCV. Following incubation for 1 h at 37 °C , the virus–antibody mixture was added to confluent MDBK cells in 24-well (16 mm diameter) microtitre dishes (Costar). The virus–antibody mixture was allowed to react with the cells for 1 h before washing and overlaying with fresh medium containing 1% FBS. Seven days later, the monolayers were fixed and stained with 5% crystal violet in 85% methanol, and the endpoint of $TCID_{50}$ was determined as described previously (Deregt *et al.*, 1989).

Results

Construction of E3 replacement recombinant Ad

An infectious recombinant Ad5 was constructed by replacing the E3 locus of the Ad genome with the BCV HE gene under the control of the SV40 early gene promoter. Two main steps were followed in the construction of recombinant Ad: subcloning of the HE gene into an Ad5 transfer vector and rescue of the hybrid transcription unit into the infectious Ad5 genome. The Ad5 transfer vector, pFGdX1, contains the right *Bam*HI B fragment of the Ad genome (map units 59·5 to 100) with a deletion of the *Xba*I D fragment (map units 78·5 to 84·7) which represents most of E3. First, the entire BCV HE gene was inserted into the *Bam*HI site of pSV2X3, then the expression cassette consisting of the HE gene flanked by the SV40 early promoter and SV40 poly-adenylation signal was excised and subcloned into the *Xba*I site of pFGdX1. The BCV HE gene was cloned in the same orientation as the E3 gene it replaced (Fig. 1).

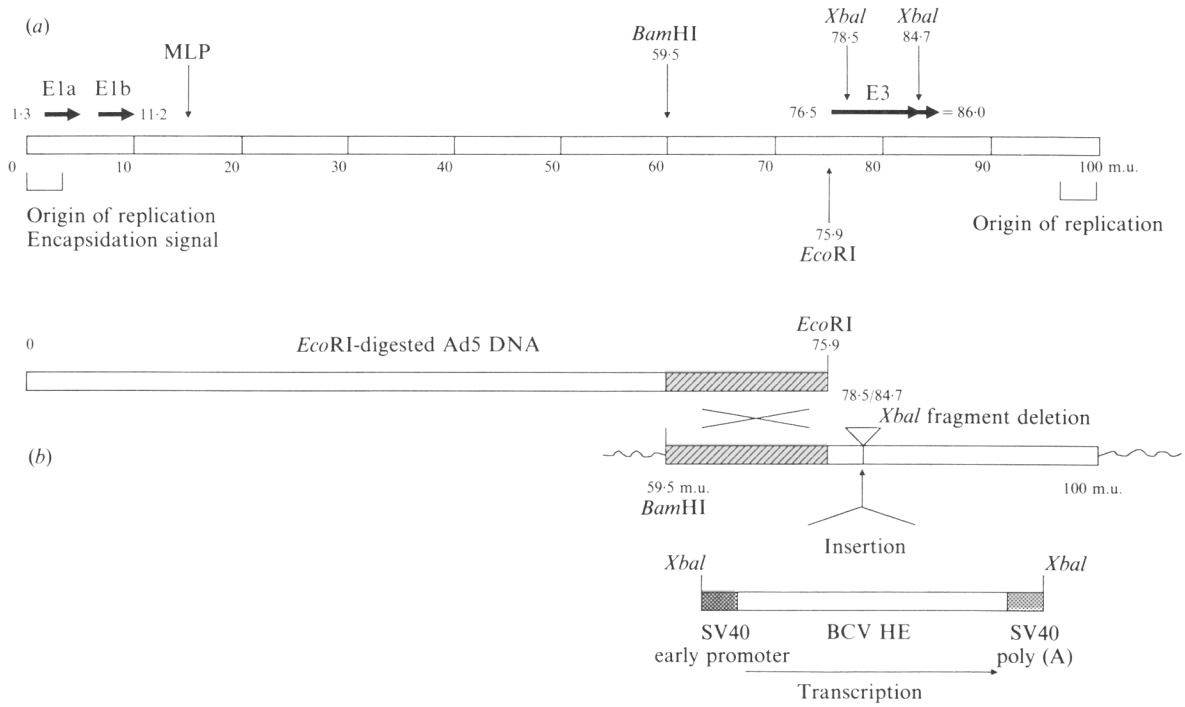

Fig. 1. Construction of helper-independent recombinant Ad. (*a*) A simplified map of the Ad5 genome. The right *Bam*HI fragment of the viral genome (map units 59·5 to 100) was cloned into pBR322, and an *Xba*I fragment (map units 78·5 to 84·7) representing most of the E3 region (map units 76·5 to 86) was deleted by *Xba*I digestion and religation, resulting in transfer vector pFGdX1 (Haj-Ahmad & Graham, 1986). The location of the E1 region often utilized for construction of defective recombinant virus is also shown at the left end of the genome. (*b*) A strategy for the construction of AdBcHE recombinant Ad. The BCV HE gene was cloned into pSV2X3 and the expression cassette consisting of the SV40 promoter, HE gene and polyadenylation signal was inserted into the *Xba*I site of pFGdX1. The resulting transfer vector was used to cotransfect 293 cells with *Eco*RI-digested viral DNA. The HE transcription unit was rescued into the viral genome by homologous *in vivo* recombination. Recombinant viruses were screened by DNA restriction endonuclease analysis. A recombinant virus having the desired structure was purified by two rounds of plaque assay.

The transfer plasmid was linearized by digesting with *Bam*HI, and the HE gene was inserted into the genome of Ad by cotransfecting 293 cells with this plasmid and Ad5 DNA digested with *Eco*RI. A recombinant virus, designated AdBcHE, was identified by restriction analysis of the resultant plaques and isolated by two more rounds of plaque purification. Purified AdBcHE was amplified to produce a virus stock which was stored at −80 °C, and used for subsequent studies. The recombinant virus remained stable upon further amplication and grew to high titres, $>10^9$ p.f.u./ml, similar to those of wild-type Ad5.

Expression, dimerization and glycosylation of the HE polypeptide

Synthesis of the BCV HE polypeptide was initially determined in human cells by immunoprecipitation and SDS–PAGE of the lysates prepared from AdBcHE-infected 293 cells. Cells were infected with approximately 10 p.f.u./cell of either recombinant AdBcHE or wild-type Ad5, and were labelled with [^{35}S]methionine at 12 h post-infection for 6 h. The BCV HE gene is capable of encoding a 45K polypeptide (Parker *et al.*, 1989), which is then post-translationally modified by glycosylation to produce a 65K polypeptide (King & Brian, 1982). In the presence of 2-mercaptoethanol, anti-BCV polyclonal rabbit serum identified a 65K polypeptide from cells infected with AdBcHE (Fig. 2*a*, lane 3). This polypeptide comigrated with the authentic HE protein isolated from BCV virions (Fig. 2*b*, lanes 1 and 3).

The fully mature HE glycoprotein found in BCV is a 130K polypeptide, a homodimer of the 65K monomer (King *et al.*, 1985). To determine whether the 65K polypeptide synthesized by recombinant Ad was capable of dimerizing, samples were immunoprecipitated and resolved under non-reducing conditions. In the absence of 2-mercaptoethanol, a 130K polypeptide was identified (Fig. 2*b*, lane 6), whereas little or no 65K polypeptide (lane 6) was seen, indicating that the 130K polypeptide was a dimer of 65K monomers. Comigration of the 65K and 130K polypeptides with the corresponding authentic HE proteins (Fig. 2*b*, lanes 1 and 3, and lanes 4 and 6) suggests that the recombinant HE is properly post-translationally modified.

Synthesis of the HE polypeptide was also examined in

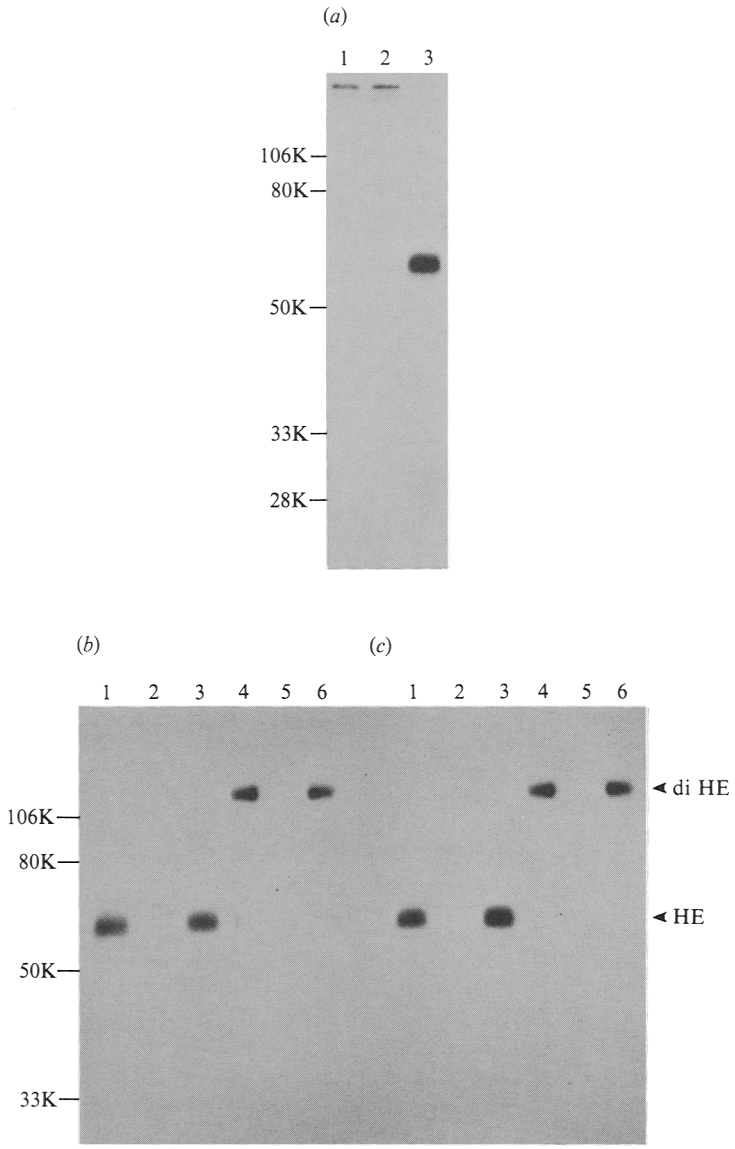

Fig. 2. (*a*) Expression of the HE polypeptide. 293 cells were mock-infected (lane 1), or infected with wild-type Ad (lane 2) or recombinant Ad AdBcHE (lane 3) at a multiplicity of 10, and labelled with 50 μCi/ml [^{35}S]methionine from 12 to 18 h post-infection. Radiolabelled cell lysates were extracted and cytoplasmic fractions were immunoprecipitated with rabbit anti-BCV antiserum using Protein A-coupled Sepharose beads. The immune complex was dissociated by boiling for 5 min in the presence of 2-mercaptoethanol, and resolved by electrophoresis on a 10% SDS–polyacrylamide gel, followed by fluorography. (*b* and *c*) Dimerization of the HE polypeptide synthesized in 293 cells (*b*) and MDBK cells (*c*). Cells were infected with wild-type Ad (lanes 2 and 5) or with recombinant Ad (lanes 3 and 6). Cells were radiolabelled with [^{35}S]methionine at 12 to 18 h post-infection. For the BCV HE as control (lanes 1 and 4), MDBK cells were infected with BCV and the virus was prepared from the supernatant by sucrose gradient centrifugation. Cell lysates or BCV virions were immunoprecipitated and the immune complexes were dissociated in the absence (lanes 1 to 3) or presence (lanes 4 to 6) of 2-mercaptoethanol followed by SDS–PAGE and fluorography. Monomeric and dimeric HE polypeptides are indicated as HE and di HE, respectively.

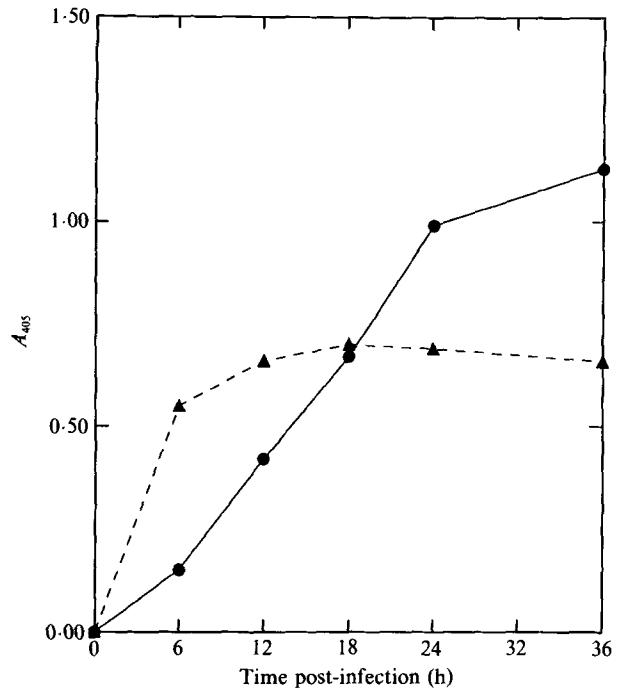

Fig. 3. Kinetic analysis of HE synthesis. MDBK cells were infected with BCV (●) or AdBcHE (▲) at a multiplicity of 10. At various times post-infection, cells were lysed and cytoplasmic fractions representing approximately 5 × 10^5 cells were diluted 1:100 in 0·85% saline, 10 mM-PBS, 0·05% Tween 20. The amount of HE antigen synthesized was determined by sandwich ELISA (Methods) using a pool of HE-specific MAbs, and is presented as the A_{405} with a reference wavelength of 490 nm.

a bovine cell line to determine the potential of using the recombinant Ad as a vector in cattle (Fig. 2*c*). MDBK cells infected with wild-type Ad5 or the AdBcHE recombinant readily developed extensive cytopathology and produced high titres of infectious virus, comparable to those seen in the human cell line. When the synthesis of HE was examined in MDBK cells, both monomeric (Fig. 2*c*, lane 3) and dimeric (Fig. 2*c*, lane 6) forms of the HE polypeptides, identical in migration rates to those found in 293 cells (Fig. 2*b*, lanes 3 and 6), were specifically immunoprecipitated, suggesting the efficient replication of recombinant human Ad in bovine cells.

To examine the kinetics of HE synthesis, MDBK cells were infected with BCV or AdBcHE at a multiplicity of 10 p.f.u./cell. At the indicated times post-infection, cell lysates were prepared and intracellular HE was measured by an antigen-capture sandwich ELISA (Fig. 3). The amount of BCV HE increased gradually but continuously in MDBK cells throughout the infection. In contrast, recombinant HE was produced very rapidly, such that at 6 h post-infection most of the maximum amount of antigen was present, with only a slight increase thereafter. The amount of HE synthesized by AdBcHE was estimated to be approximately 60% of that synthesized by BCV.

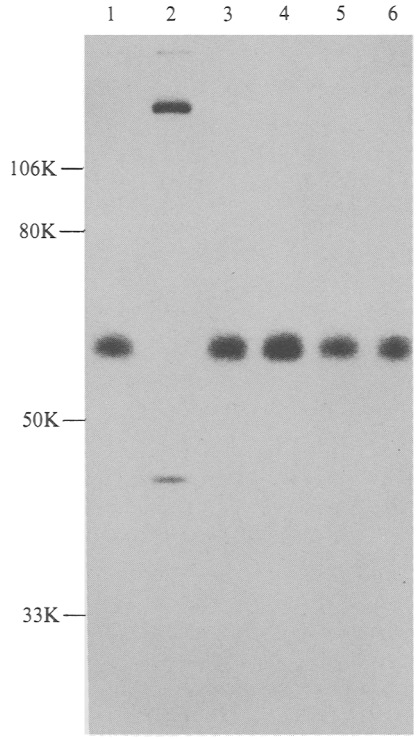

Fig. 4. Immunoreactivity of the HE polypeptide with MAbs that recognize conformation-dependent domains. AdBcHE-infected MDBK cells were labelled with 50 μCi/ml [^{35}S]methionine. The cell lysates were immunoprecipitated with MAbs specific for the BCV HE protein. Lanes 1, rabbit anti-BCV polyclonal antibody; lane 2, no antibody; lane 3, HC10-5 (antigenic group A1); lane 4, KD9-40 (group A2); lane 5, KC4-3 (group B); lane 6, BD9-8C (group C).

Glycosylation of the recombinant HE polypeptide was examined by labelling virus-infected MDBK cells with [^{3}H]glucosamine from 16 to 22 h post-infection. In the presence of a reducing agent, a radiolabelled 65K polypeptide was immunoprecipitated from cells infected with either BCV or AdBcHE, but was absent from mock-infected or wild-type Ad-infected cells (data not shown). This confirmed that the recombinant HE polypeptide produced by AdBcHE was glycosylated and indistinguishable in migration rate from the authentic BCV HE protein.

Immunoreactivity of the recombinant HE with conformation-dependent MAbs

Three BCV HE antigenic domains have been identified previously (Deregt & Babiuk, 1987). These antigenic domains are involved in eliciting BCV-neutralizing antibodies and are all conformation-dependent because pretreating the HE with reducing agents such as 2-mercaptoethanol abolishes immunoreactivity with the MAbs. We examined the reactivity of the recombinant HE polypeptide with these MAbs by immunoprecipitation. MAbs HC10-5, KD9-40, KC4-3 and BD9-8C, representing antigenic groups A1, A2, B and C, respectively (Deregt & Babiuk, 1987), all efficiently immunoprecipitated the HE polypeptide (Fig. 4, lanes 3, 4, 5 and 6). This indicates that the recombinant HE is folded in a conformation similar to that of the authentic molecule produced in BCV infections, and that it retains the important immunological epitopes.

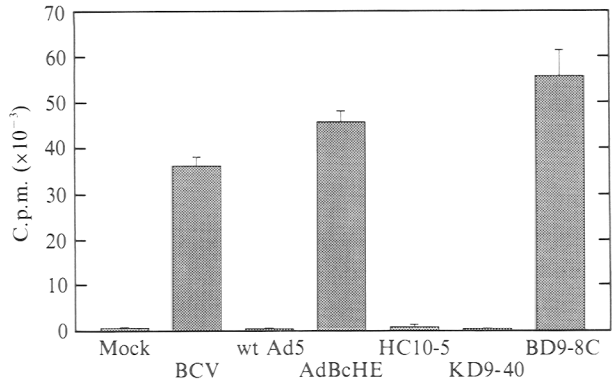

Fig. 5. Inhibition of haemadsorption activity of the HE polypeptide with group A MAbs. MDBK cells infected with Ad were pre-incubated with MAb for 1 h. Mouse red blood cells were radiolabelled with ^{51}Cr and added to the cell monolayer for the haemadsorption assay. After 10 min incubation, monolayers were washed five times to remove unbound erythrocytes. Erythrocyte–MDBK cell complexes were lysed with lysis buffer and radioactivity was determined.

Haemadsorption and acetylesterase activities

Haemadsorption activity of the recombinant HE polypeptide was determined by measuring the erythrocyte-binding activity of cells infected with AdBcHE. MDBK cells were infected with either AdBcHE or wild-type Ad5 and, at 24 h post-infection, cell monolayers were washed with PBS followed by incubation with mouse erythrocytes. Both BCV- and AdBcHE-infected cells bound erythrocytes, whereas mock-infected or wild-type Ad5-infected cells did not react with erythrocytes (Fig. 5). When virus-infected cells were pre-incubated with the HE-specific MAbs, HC10-5 and KD9-40 completely inhibited erythrocyte binding, whereas BD9-8C did not (Fig. 5). The results from the haemadsorption assays also suggest that the recombinant HE polypeptide is transported to the cell surface. Cell surface expression of the HE polypeptide was confirmed by staining the cells with FITC-conjugated antibody (data not shown).

It has been reported that the BCV HE is an enzyme that hydrolyses the ester linkage of 9-*O*-acetylated neuraminic acid (Vlasak *et al.*, 1988*a*, *b*). We examined the acetylesterase activity of the recombinant BCV HE polypeptide. Lysates prepared from virus-infected cells were incubated with bovine submaxillary mucin, and the

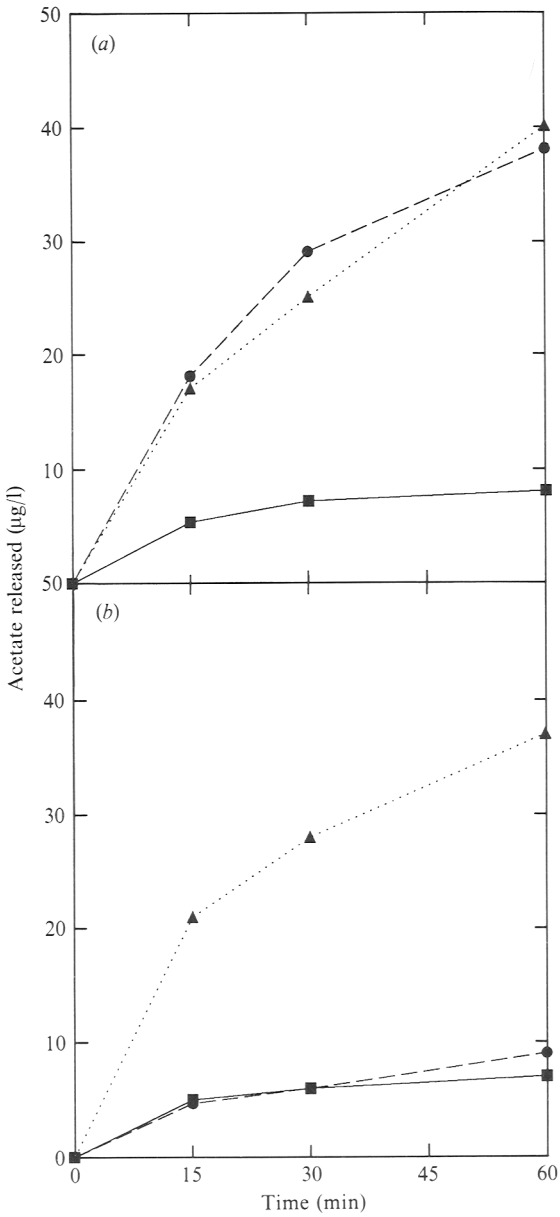

Fig. 6. (*a*) Acetylesterase activity of HE. Lysates prepared from MDBK cells infected with AdBcHE (●), wt Ad5 (■) or BCV (▲) were incubated with 25 mg of bovine submaxillary mucin for various times. Esterase activity of the recombinant HE polypeptide was determined by measuring the amount of acetic acid released into the reaction. Acetic acid release was quantified by u.v. absorption using a commercial kit. (*b*) Inhibition of the acetylesterase activity of the recombinant HE by BCV group A MAbs. Prior to addition of the mucin substrate, cell lysates were pre-incubated for 1 h with a 1:100 dilution of KD9-40 (■), BD9-8C (▲) or HC10-5 (●).

acetate released from the mucin was measured as acetylesterase activity. Both BCV- and AdBcHE-infected cell lysates exhibited acetylesterase activity, and the amount of acetate converted from the substrate increased with incubation time (Fig. 6*a*). When the cell lysates were pre-incubated with each of the above MAbs,

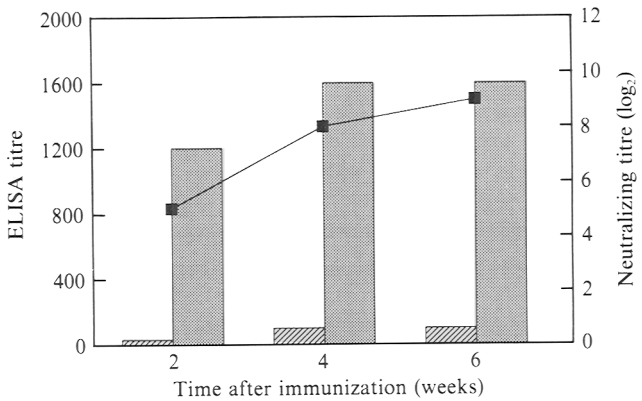

Fig. 7. Antibody response to HE polypeptide in mice inoculated with AdBcHE. Three-week-old mice were inoculated intraperitoneally with AdBcHE at 10^8 p.f.u./animal (■). Antibody responses to the HE polypeptide were measured using purified BCV by ELISA and serum neutralization assay as described in Methods. Bars represent mean ELISA titres from five mice and the line indicates the serum neutralizing antibody titre. Heat-inactivated AdBcHE was used as the control (▨).

HC10-5 and KD9-40 inhibited esterase activity whereas BD9-8C did not (Fig. 6*b*). Inhibition of both acetylesterase activity and haemadsorption activity by the same BCV-neutralizing MAbs suggests that the antigenic domain(s) recognized by both of these antibodies may be identical to or are closely associated with the domains responsible for acetylesterase activity and haemadsorption activity.

Induction of anti-HE antibody response in mice infected with live Ad

To evaluate the immunogenicity of the HE glycoprotein encoded by the recombinant Ad, 3-week-old mice were injected intraperitoneally with 10^8 p.f.u. AdBcHE, which had been purified in a CsCl gradient. The animals were bled periodically and analysed by ELISA and serum neutralizing assay. Antibodies to the BCV HE glycoprotein were detectable in animals infected with AdBcHE after 2 weeks and titres increased until 6 weeks post-infection (Fig. 7). This increase was particularly evident when the sera were analysed for the presence of virus-neutralizing antibody.

Discussion

Human Ad has been utilized widely to express proteins from various eukaryotic genes. In most cases, foreign genes have been inserted into E1 of the Ad genome, resulting in the production of a replication-defective recombinant virus. This approach limits the replication of the recombinant virus mostly to 293 cells, which constitutively express the E1-encoded proteins. We

constructed a helper-independent recombinant Ad by inserting the gene into E3. Most of the E3 region of the Ad genome was deleted and replaced with the BCV HE gene. The recombinant virus was infectious and replicated efficiently in MDBK cells as well as in 293 cells. It will be of interest to examine whether the recombinant Ad5 can replicate in cattle as efficiently as in MDBK cells. Despite the lower level of expression of the HE polypeptide by AdBcHE than by BCV (Fig. 3), we demonstrated that mice inoculated with the live recombinant virus developed a significant neutralizing antibody response to the HE protein, illustrating the potential of using Ad as a vectored vaccine. Although the anti-HE antibody titre in mice inoculated with AdBcHE was somewhat lower than we expected, this could be explained by the fact that mice are considered to be semi-permissive for Ad5 replication (Prevec *et al.*, 1989, 1990). Further information regarding antibody responses induced by Ad in cattle should provide an important approach for developing a live virus vector in this species.

In Ad infection, viral RNA synthesis from the E3 promoter is detectable at 2 h post-infection and reaches maximum level by 4 h post-infection. Transcription from the major late promoter (MLP) is initiated with the onset of viral DNA replication at 6 to 8 h post-infection, and attains a maximum level at 18 h (Ginsberg, 1984). We found that the synthesis of the HE polypeptide by the recombinant Ad in MDBK cells was detectable at 3 h post-infection. The amount of HE synthesized in MDBK cells by AdBcHE reached a significant level by 6 h post-infection and a maximum by 12 h (Fig. 3). Since the Ad kinetics are somewhat slower in MDBK cells (unpublished observation), it is suggested that the synthesis of the HE polypeptide is initially mediated by early transcription. Although the BCV HE gene was placed between the SV40 promoter and poly(A) signal (Fig. 1), the exogenous promoter may not contribute significantly, if at all, to HE gene expression. This is consistent with the expression of several foreign genes inserted into the E3 region, which were dependent for expression upon being orientated parallel to the direction of transcription from the Ad5 E3 promoter (Haj-Ahmad & Graham, 1986; Schneider *et al.*, 1989; Prevec *et al.*, 1990). Therefore, it is likely that HE synthesis in AdBcHE-infected cells is mediated largely by the Ad5 E3 promoter. In addition, because HE was still being synthesized at 16 h post-infection (unpublished data; Schneider *et al.*, 1989), the MLP may also be involved in HE synthesis, by taking over transcription from the E3 promoter at later stages of infection. However, once HE reached a maximum level, at 12 h post-infection, it remained constant until 36 h (Fig. 3). Thus, the MLP contribution to HE synthesis seems minor.

The recombinant HE polypeptide synthesized by Ad was indistinguishable from the authentic HE protein produced by BCV in both biochemical and biological properties. The recombinant HE is glycosylated and dimerized, and retains its conformational neutralizing epitopes. The HE polypeptide also maintains haemagglutination and acetylesterase activities. We have found previously that the HE-specific MAbs which neutralize BCV infectivity also inhibit both the haemagglutination and acetylesterase activities of the purified virions (Parker *et al.*, 1989, 1990*a*). Using the recombinant HE, we have demonstrated directly that neutralization of BCV by MAbs coincides with inhibition of both the haemagglutination and acetylesterase activities, suggesting that antigenic group A of the HE protein is a domain responsible for these two biological activities. It also suggests that the two enzymatic properties are closely associated in the recombinant BCV HE molecule. Detailed characterization of the functional domains for these biological activities remains to be completed.

Although E3 has been shown to be dispensable for growth of Ad *in vitro*, this region is thought to be involved in the modulation of cellular immunity *in vivo* (for a review see Wold & Gooding, 1991). An E3-encoded glycoprotein, gp19K, resides in the endoplasmic reticulum (Paabo *et al.*, 1987) and binds to major histocompatibility complex class I antigens (Kvist *et al.*, 1978), preventing their transport to the surface of virus-infected cells. This suggests that gp19K interferes with virus-specific cytotoxic T lymphocyte lysis of virus-infected cells (Rawle *et al.*, 1989). In addition, other E3-encoded proteins of 14·7K and 10·4K/14·5K protect Ad-infected cells from tumour necrosis factor (Gooding *et al.*, 1988, 1991; Wold & Gooding, 1989). It seems that one of the major roles of E3 is to allow Ad to evade host immune surveillance, and this may account for the establishment of latent or persistent infection. Thus, it is possible that recombinant Ad vectors with deleted E3 functions may avoid the possible complication of establishing latency or persistence. Nevertheless, Ad recombinants deleted in the E3 sequences have been reported to replicate in animals and the recombinants persist long enough to elicit an antibody response (Fig. 7; Morin *et al.*, 1987). In contrast, Ginsberg *et al.* (1989) have found that an E3-deleted Ad5 mutant replicates to the same extent as wild-type virus in the lungs of animals infected intranasally, and it causes considerably greater infiltration of uninfected lung tissues by lymphocytes and monocytes. Owing to these data, it has been suggested that deletion of the E3 coding sequences can alter Ad disease pathogenesis (Wold & Gooding, 1991). These factors must all be considered when designing live Ad vectors for animals so that one gains the advantages of Ad as a vector without creating any unwanted side effects.

The authors thank Brian Christie and John Rudy for their technical assistance, and Barry Caaroll for care and handling of the animals. This work was supported by the Natural Sciences and Engineering Research Council of Canada and the Medical Research Council of Canada. Published as journal series no. 138 with the permission of the director of VIDO. D.W.Y. is the recipient of a fellowship from the M.R.C. of Canada, and F.L.G. is a Terry Fox Cancer Research Scientist.

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(*Received 18 February 1992; Accepted 10 June 1992*)