*J. gen. Virol.* (1989), **70**, 993–998. *Printed in Great Britain*

Key words: *bovine coronavirus/monoclonal antibodies/E1, E2 glycoproteins*

# Monoclonal Antibodies to Bovine Coronavirus Glycoproteins E2 and E3: Demonstration of *in vivo* Virus-neutralizing Activity

By DIRK DEREGT,[1]† GLEN A. GIFFORD,[2] M. KHALID IJAZ,[3] TRENT C. WATTS,[3] JAMES E. GILCHRIST,[2] DEBORAH M. HAINES[1] AND LORNE A. BABIUK[1,3]*

[1]*Department of Veterinary Microbiology, University of Saskatchewan,*
[2]*Biostar, Inc., P.O. Box 1000, Sub P.O. 6 and* [3] *Veterinary Infectious Disease Organization,*
*124 Veterinary Road, Saskatoon, Saskatchewan, Canada, S7N 0W0*

(*Accepted 18 November 1988*)

## SUMMARY

Six monoclonal antibodies (MAbs) to bovine coronavirus (BCV, Quebec isolate) E2 and E3 glycoproteins which were found previously to be neutralizing *in vitro* were examined for virus-neutralizing activity *in vivo*. Surgically ligated intestinal loops of newborn colostrum-deprived calves were virus-inoculated, mock-infected or inoculated with a mixture of virus and antibody. Of the six BCV-specific MAbs, four were found to be protective against a virulent field isolate of BCV, as indicated by a reduction in villous atrophy. These MAbs were specific to antigenic domain A and antigenic domains A1 and A2 on the E2 and E3 glycoproteins respectively. MAbs to antigenic domains B and C on the E2 and E3 glycoproteins, respectively, were not protective.

Bovine coronavirus (BCV) is considered to be one of the leading causative agents of viral enteritis in newborn calves (Babiuk *et al.*, 1985). The disease is characterized by diarrhoea and severe dehydration and is often fatal (Mebus, 1978; Mebus *et al.*, 1973). BCV infects epithelial cells of the jejunum, ileum and colon resulting in severe shortening of villi (Mebus *et al.*, 1973). The virion is composed of four structural proteins, a nucleocapsid (N) protein and three envelope glycoproteins that have been designated E1, E2 and E3 (Storz *et al.*, 1981; King & Brian, 1982; Deregt *et al.*, 1987). Monoclonal antibodies (MAbs) to the Quebec isolate of BCV have been produced and virus-neutralizing (*in vitro*) antibodies were found to be directed to the E2 glycoprotein (gp190/gp100) and the disulphide-linked dimer glycoprotein E3 (gp124) (Deregt & Babiuk, 1987).

Our interest in the possible employment of subunit or synthetic oligopeptide vaccines for the prevention of coronavirus-induced neonatal calf diarrhoea has led us to examine the roles that the E2 and E3 proteins may play in an *in vivo* infection. Specifically, we were interested in determining whether MAbs to specific epitopes on these proteins could protect calves from BCV-induced intestinal villous atrophy. Thus, to determine whether anti-BCV MAbs that were neutralizing *in vitro* could also be effective against BCV infection *in vivo*, six MAbs representing five antigenic groups (Table 1) were mixed with virus and inoculated into surgically ligated intestinal loops of newborn calves.

Colostrum-deprived Holstein calves were obtained from local dairy farms within a few h of birth. After transport, the calves were fed 2 l of a balanced electrolyte solution (Ionalyte; Rogar/STB) via an oesophagal feeder. Feeding was repeated 8 h later. Approximately 24 h after birth, the calves were anaesthetized by inhalation of a mixture of halothane, nitrous oxide and oxygen. Gentamicin sulphate (Garasol, Schering Canada) (50 mg) was added to 4·5 l of lactated

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† Present address: Animal Diseases Research Institute, P.O. Box 640, Lethbridge, Alberta, Canada, T1J 3Z4.

Table 1. *Protective effects of BCV MAbs*

| Antibody designation | Isotype | Protein specificity | Antigenic group | *In vitro* neutralization titre* | *In vivo* protection† |
|---|---|---|---|---|---|
| HB10-4 | G1 | E2 | A | 12500 | + |
| JB5-6 | G2a | E2 | A | 50000 | + |
| BB7-14 | G2b | E2 | B | 80000 | − |
| HC10-5 | G2a | E3 | A1 | 5000 | + |
| KD9-40 | G2a | E3 | A2 | 8000 | + |
| BD9-8C | G2a | E3 | C | 800 | − |
| BCV antiserum‡ | | | | ND§ | + |

* Reciprocal of the dilution of antibody (ascites fluid) that produced a 50% reduction of virus plaque number. Antibody titres were in general lower than previously reported (Deregt & Babiuk, 1987), because different ascites fluids were used in this study.

† Protection was determined by reduction of virus-induced villous atrophy in intestinal loops: +, protective; −, not protective.

‡ Antiserum was obtained from a calf that survived an experimental infection with BCV isolate no. 77 and which was subsequently immunized twice with purified BCV (Quebec isolate).

§ ND, Not done. ELISA titre was 512000.

Ringer's solution and administered by the intravenous (i.v.) route at a rate of 500 ml/h during surgery and until calves were euthanized. Intestinal gut loops were produced as described by Carpio *et al.* (1981). Beginning at a point 60 cm cranial to the ileo–caecal junction, segments of ileum and jejunum were exteriorized through a left flank incision. These segments were surgically ligated with cat gut at 10 cm intervals to create three series of intestinal loops. Ligations were done carefully to avoid interfering with the blood supply. Each series consisted of test (virus-inoculated) loops separated by uninoculated security loops. Intestinal loops were inoculated with 0·5 ml of a suspension of faeces (diluted 1/100 in Eagle's MEM; Gibco) containing a pathogenic field isolate of BCV, in combination with an equal volume of undiluted BCV-specific MAb (Table 1) or bovine herpesvirus type 1 (BHV-1)-specific MAb (ascitic fluids), BCV hyperimmune polyclonal antiserum, or MEM, after mixing for about 1 min. The virulent field isolate, designated no. 77, was obtained as a gift from Dr G. H. Woode, College of Veterinary Medicine, A & M University, College Station, Tx., U.S.A. and was ELISA-negative for bovine rotavirus. Mock-infected control loops, inoculated with MEM only, were included in each series. Each combination was replicated by inoculating loops of each series in at least three different animals for a minimum of nine trials. A total of six animals was used. Following recovery from anaesthesia, a narcotic analgesic [Temgesic (Buprenorphine); Reckitt and Colman] was administered intramuscularly to each calf at 8 h intervals to relieve abdominal discomfort. Calves were euthanized by an i.v. overdose of sodium pentobarbital 24 h after virus inoculation of intestinal loops. All experimental procedures conformed to the Canadian Council of Animal Care guidelines.

After euthanasia, tissues from intestinal loops were examined visually, and fluids that accumulated were measured and subsequently assayed for the presence of BCV antigen by a capture ELISA (Crouch *et al.*, 1984) using a BCV N-specific MAb. Virus-induced villous atrophy was assessed by measuring the length of intestinal villi from fixed and stained tissues with an ocular micrometer. Ten intestinal villi for each test loop were measured to calculate the mean villus length (tip of villus to villus base) for each loop.

Upon gross examination, intraluminal tissues from virus-inoculated (virus plus MEM) intestinal loops were observed to be denuded and smooth compared to the healthy, corrugated appearance of intestine from mock-infected loops. Further, the intraluminal surfaces were often congested in virus-inoculated loops. The quantity of fluid (brown or straw-coloured) that had accumulated within virus-inoculated loops greatly exceeded that which had accumulated in mock-infected loops (Fig. 1). Intestinal villi appeared normal in tissues from mock-infected loops by histological examination with a mean villus length of 521 ± 23 (±S.E.M.) µm (Fig. 1 and 2*a*). In contrast, the epithelium from virus-inoculated loops was necrotic and the intestinal villi

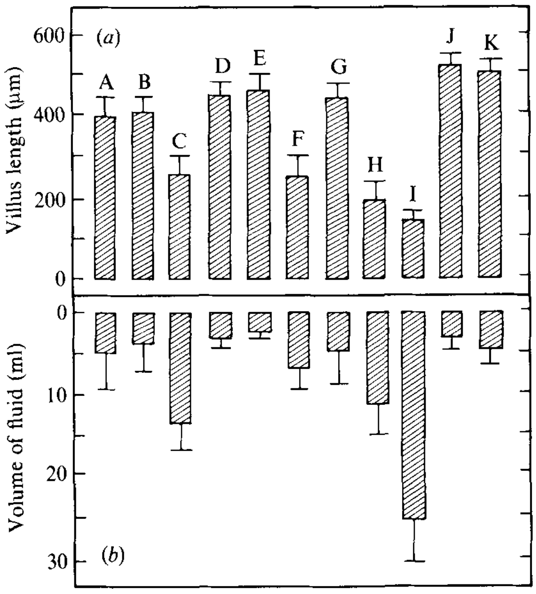
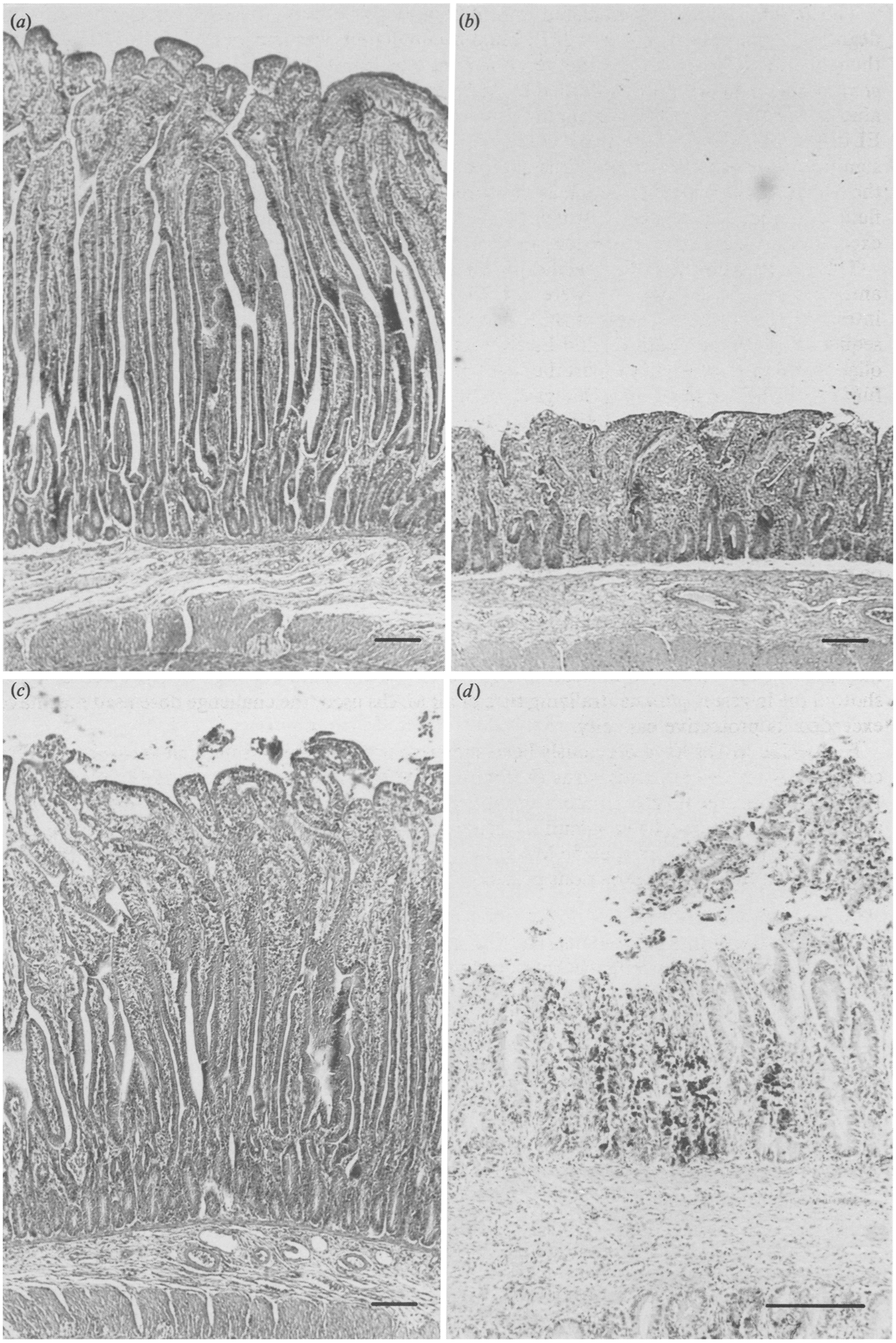


Fig. 1. The effect of BCV E2- and E3-specific MAbs on virus-induced pathological changes in calf intestinal loops. (*a*) The mean length (S.E.M. indicated) of villi from intestinal loops for each treatment; (*b*) the mean volume (S.E.M. indicated) of fluid accumulated in intestinal loops for the same treatments. The treatments (virus and antibody) and the number of calves (c) and intestinal loops (l) tested for each treatment were: A, E2-specific MAb HB10-4 (c = 3, l = 9); B, E2-specific MAb JB5-6 (c = 3, l = 9); C, E2-specific MAb BB7-14 (c = 6, l = 17); D, E3-specific MAb HC10-5 (c = 6, l = 17); E, E3-specific MAb KD9-40 (c = 5, l = 15); F, E3-specific MAb BD9-8C (c = 3, l = 9); G, bovine BCV antiserum (c = 3, l = 9); H, anti-BHV-1 MAb (c = 3, l = 9); I, virus-inoculated (plus MEM), no antibody (c = 3, l = 11); J, mock-infected (MEM only) (c = 6, l = 31); K, no injection (c = 2, l = 12). H and I were virus controls in different sets of calves. Villus lengths except for C, F and I were statistically different from H (Kruskal-Wallis one-way analysis of variance, $P < 0.01$).

were severly shortened with a mean villus length of only 135 ± 21 μm (Fig. 1). Further, BCV antigen could be detected within intestinal cells from virus-inoculated control loops, indicating that these cells were infected with virus (Fig. 2*d*).

To determine whether anti-BCV MAbs that had been previously found to be neutralizing *in vitro* could also neutralize BCV infectivity *in vivo*, virus inoculum was briefly mixed with MAbs and injected into intestinal loops, and the results were compared with virus-inoculated and mock-infected intestinal loops. Further, BCV polyclonal antiserum and anti-BHV-1 MAbs were also mixed with virus and injected into intestinal loops to serve as controls. Four of the six BCV-specific MAbs, E2-specific HB10-4 and JB5-6 (both antigenic group A) and E3-specific HC10-5 (group A1) and KD9-40 (group A2) were found to be protective, as indicated by the villus lengths of intestinal loops after treatment with these antibodies (Fig. 1 and 2*c*). Villus lengths in the intestinal loops treated with virus and these four MAbs were over 85% of those in the corresponding mock-infected loops, whereas in unprotected loops they were less than 50% of the corresponding mock-infected loops. The protective effect of BCV polyclonal antiserum was similar to that of protective MAbs (Fig. 1). After these treatments tissues showed little, if any, immunochemical staining for BCV antigen (not shown). In contrast, BHV-1-specific MAbs as expected, were not protective (Fig. 1 and 2*b*, *d*). E2-specific MAb BB7-14 (group B) and E3-specific MAb BD9-8C (group C) were also not protective, as the pathological changes (necrosis and villous atrophy) observed in these treatments were similar to those of virus-inoculated control loops (virus plus MEM or virus plus BHV-1-specific MAb). Further, villus length measurements from loops inoculated with a mixture of virus and these MAbs were not statistically different from virus-inoculated control loops (Fig. 1).


(a)
(b)
(c)
(d)

The amount of fluid accumulated in intestinal loops showed a positive correlation with the degree of villous atrophy ($r = 0{\cdot}827$). Fluid accumulation was unexpected since it is generally thought that viral infections do not result in fluid movement into the lumen (reviewed in Babiuk *et al.*, 1985). The protection afforded by MAb administration was also demonstrated when the amount of cell-free antigen present in the luminal contents of intestinal loops was determined by ELISA (not shown). Intestinal contents of all virus-inoculated control loops contained significant quantities of coronavirus antigen. In contrast, test loops inoculated with BCV and the MAbs that induced protection as measured by a significant reduction of villous atrophy and fluid exudation did not contain detectable levels of cell-free antigen in the fluids, with the exception of one loop where a low level of virus antigen was present.

The results show that E2-specific MAbs of antigenic group A and E3-specific MAbs of antigenic groups A1 and A2 were similar to BCV hyperimmune antiserum in protecting intestinal villi from the effects of BCV infection. These results suggest that specific amino acid sequences present on both E2 and E3 BCV glycoproteins can be potential targets for synthetic oligopeptide vaccines and support the suggestion that the E3 protein has an important biological function in BCV infectivity (Deregt & Babiuk, 1987).

Of several possible explanations for the finding that two of the six MAbs in this study neutralized virus *in vitro* yet were not protective *in vivo*, one is that there may be a lack of conservation of epitopes on the virulent isolate employed. This was apparently the case for the epitope recognized by the E2-specific MAb, BB7-14, as later antibody-binding studies showed that this MAb did not bind the challenge virus in an ELISA. In contrast, the E3-specific MAb BD9-8C bound to this virus, indicating that the epitope recognized by this MAb was conserved on the virulent isolate (not shown). Thus it is possible that epitope C on the E3 glycoprotein may be irrelevant for the infectivity of the virus *in vivo*. Further, the virus used in this study was exposed to antibody for only a short time, compared to *in vitro* studies (1 h), before it came in contact with cells. Thus, a possible difference in the binding kinetics or avidity of MAb BD9-8C may explain the difference in neutralizing activity *in vitro* and *in vivo*. Finally, since BD9-8C showed the lowest *in vitro* neutralizing titre of all MAbs used, the challenge dose used may have exceeded its protective capacity.

E2-specific MAbs have previously been shown to protect against infection *in vivo* by another coronavirus, mouse hepatitis virus (Talbot *et al.*, 1984; Buchmeier *et al.*, 1984; Wege *et al.*, 1984). However, the E3 glycoprotein, identified as the haemagglutinin protein (King *et al.*, 1985) appears to be unique to haemagglutinating mammalian coronaviruses (Hogue *et al.*, 1984). The protective effect of two E3-specific MAbs *in vivo* in this study emphasizes the importance of the E3 protein in BCV–cell interactions previously indicated by *in vitro* virus neutralization studies.

This work was supported by grants from the Natural Sciences and Engineering Research Council, the Medical Research Council and Farming for the Future (Alberta). Published with permission of the director as journal series no. 70.

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Fig. 2. The reduction of virus-induced pathological changes to villi by a representative anti-BCV MAb. (*a*, *b*, *c*) Light micrographs taken from tissues from intestinal loops. Tissues were fixed in Bouin's solution and stained with haematoxylin and eosin. These tissues were collected from loops of the same series in the lower jejunum of calf 87-015. Treatments were as follows: (*a*) mock-infected; (*b*) virus plus anti-BHV-1 MAb; (*c*) virus plus E3-specific MAb KD9-40. (*d*) Immunohistochemical localization of BCV antigen in intestinal epithelium from a control loop (virus plus anti-BHV-1 MAb). For immunochemical staining, Bouin's fixed tissues were briefly digested with 0·1% protease (Type XIV; Sigma), treated with 0·15% $H_2O_2$ in methanol to inactivate endogenous peroxidase and blocked with 5% normal rabbit serum in Tris-buffered saline (TBS) before incubation with a mixture of anti-BCV MAbs. After subsequent incubation with biotin-labelled antiserum to mouse IgG, tissue sections were incubated with avidin–biotin–peroxidase solution (Vectastain ABC; Vector Laboratories). Tissues were stained by incubation in 1 mg/ml of 3,3′-diaminobenzidine (Electron Microscopic Products) in TBS and 0·5% $H_2O_2$ and subsequently counterstained with haematoxylin. All bar markers represent 200 μm.

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(*Received 7 July 1988*)