Production of Monoclonal Antibodies against the Kakegawa Strain of Bovine Coronavirus and Their Characterization

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Bovine coronavirus (BCV) is an enteric virus which causes diarrhea in both neonatal calves [2, 9, 10] and adult cattle [4, 11]. Isolation of BCV from diarrheal feces in tissue cultures (TC) is very difficult. Diagnosis of BCV infection has been conducted by the detection of increased antibody. We developed the direct antigen detection from feces using specific antibody. The present study describes the production and characterization of monoclonal antibodies against the TC-adapted Kakegawa strain of BCV [1]. The technique of monoclonal antibody production utilized was the procedure reported by Köhler & Milstein [8]. The Kakegawa strain of BCV was used for immunizing mice. The virus was grown in BEK-1 cells [1] and purified by a sucrose density gradient centrifugation method described elsewhere [1] in order to use the virus as immunizing and enzyme-linked immunosorbent assay (ELISA) antigens. BALB/c mice received two injections of 2 μg protein/m*l* of purified virus in complete Freund's adjuvant first subcutaneously, then intraperitoneally, 2 weeks apart. Three weeks later, the mice were injected intravenously with the same dose of purified virion without adjuvant. Three days after the last immunization, spleen cells were fused to P3-X63-Ag8-U1 myeloma cells using polyethylene glycol #4000 (Merck & Co., Inc. U.S.A.).

Growth medium (GM) for the hybridoma was Eagle's minimum essential medium containing 10% fetal bovine serum, L-glutamine (2 mM), 1% vitamin solution (GIBCO Laboratories, U.S.A.), penicillin (100 U/m*l*), and streptomycin (100 μg/m*l*). Hybridomas were selected with HAT medium containing hypoxanthine, aminopterin and thymidine. Secretion of specific antibody against BCV was determined by ELISA. ELISA was performed as follows. One hundred microliters of antigen suspension containing 2 μg/m*l* in carbonated buffer (pH 9.6) were distributed to each well of a 96 well ELISA plate (Greiner, W. Germany) at 4°C overnight, following which each well of antigen-coated plates was treated with phosphate buffered saline (PBS) containing 0.05% Tween 20 (Tween-PBS) and 0.5% egg albumin (Difco Laboratories, U.S.A.) at room temperature for 1 hr. The plates were stored at −80°C until use. Before use, the plates were washed 3 times with Tween-PBS, and then 50 μ*l* of a test sample were distributed into each well. After incubation at 37°C for 1 hr, the plates were washed 3 times with PBS, 50 μ*l* of anti-mouse immunoglobulin (Ig) rabbit serum conjugated to horse radish peroxidase (Cappel Laboratories, U.S.A.) applied and the plates were incubated at 37°C for 1 hr. The plates were then washed 3 times as before, and 100 μ*l* of a substrate solution containing 1.46 mM 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) and 0.28 mM hydrogen peroxide (pH 4.0) were applied to each well. After incubation at room temperature for 40 min, the optical density at 415 nm was measured. After screening, hybridomas which showed an optical density reading of more than 0.2 were cloned twice using microcapillaries, and antibody secretion was reexamined by ELISA. To obtain partially purified antibody, hybridomas were grown in GM without serum and the resulting supernatants concentrated twice by 50% saturation with ammonium sulfate and then dialysed by PBS. The final volume was one-twentieth that of at the start. These concentrates were assayed for antibody activities by hemagglutination inhibition (HI) and neutralization (NT) tests [1]. Classification of Ig was determined by the MONOAB-ID kit (Zymed Laboratories, U.S.A.).

Twelve hybridoma clones were established as a result of three fusions. Seven of the clones secreted IgG_1 and four IgM with the final one unidentified (Table 1).

Only two (4H4, 7F5) of the twelve clones showed both HI and NT activities (Table 1). Although these two clones exhibited a high titer against BCV in both the ELISA and HI tests, they showed differential reactivities in the NT test (Table 1).

Table 1. Characteristics of BCV monoclonal antibodies

Clone designation	Immunoglobulin Isotype	Reciprocal titer ELISA[a)]	HI[b)]	NT[c)]
1D11	IgG_1	3,200	<2	<1
2C8	IgG_1	6,400	<2	<1
2G5	—	10	<2	<1
4E6	IgM	10	<2	<1
4H4	IgG_1	6,400	2,084	>4,096
5A10	IgM	200	<2	<1
5H4	IgG_1	800	<2	<1
6D5	IgM	200	<2	<1
7F5	IgG_1	6,400	1,024	256
10C2	IgG_1	800	<2	<1
10E6	IgM	10	<2	<1
10G10	IgG_1	400	<2	<1

a) Enzyme-linked immunosorbent assay: Reciprocal of the highest dilution of antibodies with an optical density reading of more than 0.5 at 415 nm.
b) Hemagglutination inhibition test
c) Neutralization test: Reciprocal of the highest dilution of antibodies which showed no cytopathic changes in at least one of two tubes.

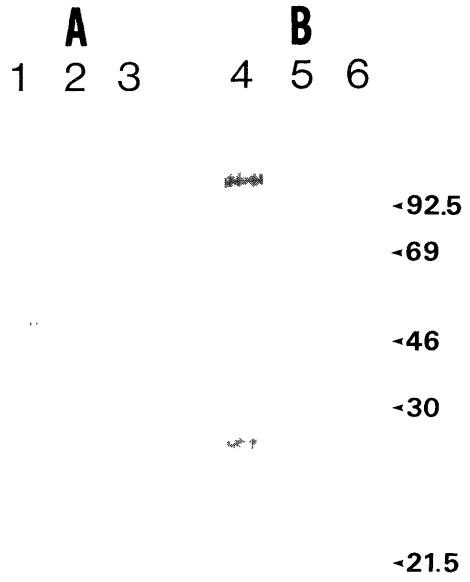

Fig. 1. A: Immunoperoxidase staining of viral polypeptides reacted with polyclonal mouse antiserum (Lane 1), monoclonal antibodies 1D11 (Lane 2) and 2C8 (Lane 3).
B: Immunoprecipitate of [^{35}S] methionine labelled virion polypeptides reacted with polyclonal mouse anti-serum (Lane 4), and monoclonal antibodies 4H4 (Lane 5) and 7F5 (Lane 6).
Samples were electrophoresed on a 10% polyacrylamide gel in the absence of 2-mercaptoethanol. Positions of molecular weight standards are indicated to the side of the gel.

These results suggest that the monoclonal antibodies 7F5 and 4H4 were specific for the surface glycoprotein of the virion [3, 7]. The specificity of the monoclonal antibodies was determined by immunoperoxidase staining and by immunoprecipitation experiments. In immunoperoxidase staining analysis, the viral polypeptides derived from purified virions as described above were transferred to nitrocellulose sheets after separation by polyacrylamide gel electrophoresis under non-reducing conditions, then reacted immunologically with monoclonal antibodies. Two monoclonal antibodies (2C8, 1D11) reacted with a polypeptide having a molecular weight (Mw) of 53 kilodalton (kDa) which seemed to be a nucleocapsid protein (Fig. 1A).

In immunoprecipitation experiments [5], the [^{35}S] methionine labeled virion lysate of BCV Kakegawa strain reacted immunologically with mouse antisera and monoclonal antibodies (Fig. 1B). Six polypeptides with Mw of 110 kDa, 100 kDa, 93 kDa, 60 kDa, 53 kDa and 23 kDa, respectively, were immunoprecipitated by the polyclonal mouse antisera. Both the 4H4 and 7F5 monoclonal antibodies precipitated the 93 kDa polypeptide. This polypeptide showed the same electrophoretic mobility under reducing conditions using 2-mercaptoethanol (data not shown).

BCV is composed of four structural proteins, a nucleocapsid protein (N) with a Mw of 52 kDa, a small transmembrane glycoprotein (M) with a Mw of 26 kDa (gp 26), a large glycoprotein (S) which forms peplomers with a Mw of 120–110 kDa (gp 120–110), and a third glycoprotein (H) with a Mw of 140 kDa (gp 140) [6]. King *et al.* [7] reported that gp 140 kDa H protein (dimeric form of gp 65) had been identified as a virion hemagglutinin in bromelain and pronase treatment experiments. However, the biological functions of BCV S and H proteins are still unclear. Monoclonal antibodies to both S and H proteins are efficient at neutralizing virus infectivity *in vitro* [3]. On the contrary, Vautherot and Laporte [12] reported that monoclonal antibodies to BCV S protein (gp 105, which is one of the S subunits with a high Mw) neutralized the infectivity and inhibited the aggulutination of rat erythrocytes by the virus. According to our data, the 93 kDa polypeptide precipitated by the 4H4 and 7F5 monoclonal antibodies did not change Mw under either reducing or non-reducing electrophoretic conditions. Thus, this 93 kDa polypeptide seems to be one of the S subunits which has a low Mw. Further studies are necessary to determine the functions of the H and S proteins of BCV, as well as the function of each S subunit.

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