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Development of monoclonal antibodies against bovine herpesvirus-1 (BHV-1)

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ABSTRACT

The BHV-1 antigen was produced in bulk by propagating BHV-1 in Madin Darby bovine kidney cell line. The antigen purified by 20–40% sucrose density gradient centrifugation was confirmed for presence of virus. The BALB/C mice were immunized by purified virus via intraperitoneal route. The spleenic lymphocytes from immunized mice were fused with myeloma cells using polyethylene glycol as a fusion agent and suspended in HAT (hypoxanthine aminopterin thymidine) medium for differentiation of hybrids. A total of 11 clones were observed, out of which 7 were positive for antibody production. The supernatant of all the positive clones showed very low concentration of antibody, except the clone $B_{6.1}$, where the concentration of antibody was higher than other positive clones. The culture supernatant of $B_{6.1}$ hybrid clone was isotyped by enzyme linked immunosorbent assay (ELISA) based isotyping kit and isotype of monoclonal antibody was of IgM class. In western blotting, the IgM monoclonal antibody identified the 27 KD protein of BHV-1.

Key words: AGPT, *Bovine Herpesvirus-1,* ELISA, Hybridoma cell culture, Monoclonal antibody.

Bovine herpesvirus-1, a DNA virus belonging to family Herpesviridae, is associated with different clinical conditions like respiratory infections, conjunctivitis, vulvovaginitis, abortion, and balanoposthitis in males. It is also associated with encephalitis and generalized systemic infections (Gibbs and Rweyemamu 1977). The virus can remain latent in the ganglia of the infected but clinically normal animals (Pastoret *et al.* 1979). Mayfield *et al.* (1983) identified 3 strains of BHV-1 namely I, II, III depending on the restriction pattern. Studdert (1989) reclassified them as BHV-1.1 (respiratory form), BHV-1.2 (genital form) and BHV-1.3 (encephalitic form). BHV-1 consists of 25 to 33 polypeptides of which 11 are glycosylated (Misra *et al.* 1981, Bolton *et al.* 1983, Gregersen *et al.* 1985) and associated with virus envelope (Misra *et al.* 1981, Bolton *et al.* 1983). The three sets of enveloped glycoproteins gI, gIII and gIV were characterized earlier which are involved in the immune response (Collins *et al.* 1984, 1985, Gregersen *et al.* 1985, Marshall *et al.* 1986, 1988, Van Den Hurk and Babuik 1986). Babiuk *et al.* (1987) showed that gIV produces the highest serum neutralizing antibody titres with gI being the least immunogenic. In India, existence of BHV-1 was first recorded by Mehrotra *et al.* (1976). The virus is found to be associated with different disease conditions in our country. The strains of BHV-1 with different tissue affinities may exist in the field conditions and these different clinical strains can be differentiated by restriction endonuclease strategies (Durhams *et al.* 1991).The monoclonal antibodies specific for the major BHV-1 glycoproteins GVP6/11a/16, GVP7, GVP3/9 and GVP11/b are used to determine the glycoproteins and identify the epitopes involved in neutralization and antibody complement lysis (Van Den Hurk *et al.* 1984). Since antigenic structure of the viruses have been successfully analysed using monoclonal antibodies therefore this study was planned to develop monoclonal antibodies against BHV-1.

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MATERIALS AND METHODS

Propagation of virus in MDBK cell culture: The virus BHV-1 isolate (IBR 216II batch no. 9/98) available in the Department of Veterinary Microbiology, PAU, Ludhiana was used in the present study. The virus was propagated in MDBK cell line using Dulbecco's minimum essential medium with 10% and 2% FCS for growth and maintenance, respectively for preparing antigen in bulk quantity.

Purification of the virus: The cell culture fluid after 3 cycles of freezing and thawing was centrifuged at 7000rpm for 20 minutes at 4°C to collect supernatant. The supernatant was subjected to ultracentrifugation at $10^5 \times g$ for 1 h to pellet the virus. The pellet was resuspended in 500µl of

phosphate buffered saline and subjected to 20–40% (w/v) sucrose density gradient centrifugation (the sucrose gradient was prepared in 200 mM tris 50 mM NaCl buffer, pH 8.0) at $10^5 \times$ g for 2 h and 30 min to collect the virus at the junction of 20–40% sucrose. The presence of virus was confirmed by agar gel precipitation test (AGPT) as per Le-Yeune *et al.* (1977) and the protein content was estimated (Lowry 1951).

Immunization of BALB/C mice: BALB/C mice of 6–7 weeks of age procured from National Institute of Nutrition, Hyderabad were used for immunization. Four hundred microgram of purified virus was mixed with equal amount of Freund's complete adjuvant to prepare an emulsion and injected into mice *via* intraperitoneal route. Booster was administered after 14 days with 100µg of purified virus alone *via* intraperitoneal route. Three days after the second injection all the mice were tail bled to collect serum which was subjected to ELISA to determine the antiviral antibodies. For this the ELISA plate was coated with purified virus (10µg/ml) and incubated overnight at 4°C. 100µl of each dilution (1:20 and 1:40) of the test serum was added in duplicate wells and the normal serum served as negative control. The plates were incubated at 37°C for 2 h. After washing the plate 3 times 100µl of anti-mouse IgG-Horse radish peroxidase conjugate (1:10000) was added in each well and further incubated the plate at 37°C for 2 h. After washing, 100 µl of substrate solution was added to each well and incubated the plate at room temperature for 10 min. After stopping the reaction with 50 µl of 1M H_2SO_4, optical density was read at 450nm wavelength.

Hybridoma cell culture

Myeloma cell line: Non-secretory myeloma cell line SP2/O/Ag-14, procured from National Centre for Cell Science, Pune was subcultured and maintained in RPMI-1640 medium with 10% foetal calf serum containing 0.2% streptopenicillin (500×) and 1% glutamine. A day prior to fusion, myeloma cells were subcultured in growth medium containing 2% 8-azaguanine. Myeloma cells growing in log phase of growth were used for fusion.

Preparation of feeder spleen cell layer: Albino mice procured from Punjab Veterinary Vaccine Institute, Punjab Agricultural University,Ludhiana were used for the preparation of feeder layer. One day prior to fusion, spleenocyte feeder cell layer was laid down in the 24 well tissue culture plate in HAT medium having 10^6 cells/ml/well. The plates were incubated in a humid incubator at 37°C with 5% CO_2 tension.

Preparation of polyethylene glycol (PEG): PEG (molecular weight 3000–4000) was autoclaved and immediately suspended in RPMI-1640 medium was added aseptically by keeping the vial at 56°C in water bath.

Fusion protocol: Myeloma cells in log phase of growth were brought into suspension by tapping and the suspension was transferred into calibrated centrifuge tube and final volume made to 10ml with serum free media. The immunized BALB/C mouse was etherized and disinfected by dipping into 70% alcohol. The spleen was removed into a petriplate and washed with 2ml of serum free media twice. The splenocytes were released into the medium by flushing with the medium into the spleen and the cells were collected by centrifugation. The splenocytes and myeloma cells were separately centrifuged at 4000 rpm for 10 min. The supernatant was discarded and pellet was brought into suspension by tapping. Equal number (10^7) of spleen cells and myeloma cells were mixed and co-pelleted by centrifugation at 4000rpm for 10 min. To the pellet, 1 ml of PEG was added slowly along the side of tube over 1 min taking care to swirl the tube gently while adding PEG so as to ensure proper mixing of PEG solution. The tube was incubated at room temperature for 1 min to facilitate fusion of myeloma and lymphocyte cells. Later PEG was diluted by adding 10ml of growth medium over 10 min and centrifuged at 4000rpm for 3min. After discarding the medium the final pellet was dissolved in 48ml of HAT medium, and the cell suspension was dispensed in a quantity of 1ml in two 24 well plates which had already been seeded with normal mice spleen feeder cells a day before. The plates were incubated at 37° C in 5% CO_2 tension. The plates were left undisturbed for 3–4 days and examined daily for signs of growth. When the hybrids started showing growth, the supernatants were collected aseptically and screened for anti-BHV-1 antibody secretion by indirect plate ELISA (Florent and De Marnaffe 1986).

Cloning: After identification of single clone ($B_{6.1}$), the cells were dislodged and ten-fold serial dilutions were made starting from 10^{-1} to 10^{-6} and dispensed in 1ml quantity into each well which had feeder layer one day before seeding.

Isotyping of antibodies: Isotyping of monoclonal antibodies in cell culture supernatants was performed with the indirect ELISA based isotyping kit.

Immunoblotting: The specificity of the monoclonal antibody for a particular protein of BHV-1 was determined by immunoblot. For this discontinuous system of polyacrylamide gel electrophoresis (PAGE; Laemelli 1970) was followed. Electrophoresis on 10% resolving gel with 4% stacking gel in tris glycine buffer was carried out at 140 volts for 3 to 4 h. Following electrophoresis, the protein was blotted to nitro-cellulose membrane (NCM) in transfer buffer. Transfer of protein was carried out in transblot apparatus at 100 volts for 1 h. A total of 600ml of infected cell culture fluid was used for purification of virus.

RESULTS AND DISCUSSION

The virus appeared at the junction of 20–40% sucrose gradient. The presence of the virus was confirmed by AGPT using known anti-BHV-1 serum. The protein content of the virus was found to be 10µg/ml.

The purified virus was used for immunization of BALB/

Table 1. Results of ELISA to screen the serum from immunized mice for the presence of antibodies

Dilutions	O D values		
	Mouse 1	Mouse 2	Normal serum
1:20	1.194	0.659	0.027
1:40	0.887	0.502	0.018

C mice. Three days after the booster injection, the mice were tail bled and the serum obtained was subjected to indirect ELISA with known positive purified BHV-1. The serum gave positive reaction in ELISA (Table 1).

Hybridoma cell culture: The hybridomas started differentiating themselves in HAT medium by seventh day post-fusion. Microscopically, they appeared round, shining cells and growing as foci in different areas of the wells. Supernatant was collected and fresh HAT medium supplemented into all the wells at every fourth day. The supernatants were regularly screened for the presence of anti-BHV-1 antibodies by indirect ELISA using mouse anti-BHV-1 antibodies and HAT as positive and negative controls. The optical density values of negative controls vary between 0.01 to 0.03 whereas; optical density values of positive controls vary between 0.75 to 0.88 at 1:40 dilution of the serum. The cut off value was taken as twice the optical density of negative control as suggested by Suresh *et al.* (1999).

The growth pattern of the hybrid cells is described in Table 2. On day 10th post-fusion, single or several shining cells were present throughout the respective wells. On day 15th, hybrid no. $A_{2.1}$ and $C_{1.1}$ showed growth in the form of small clumps of 1 to 5 whereas; in $B_{6.1}$ growth was observed as one small clump. The cells were dislodged, serial ten-fold dilution starting from 10^{-1} to 10^{-6} were made in HAT medium. Each dilution was added in one well in a quantity of 1 ml, immediately after dispensing all the wells were observed for

Table 2. Growth pattern of hybrid cells

Hybrid no.	Days, post-fusion				
	10	15	20	23	28
$A_{2.1}$	1	2	4	degenerated	-
$B_{6.1}$	1	2	-	3	degenerated
$C_{1.1}$	1	2	4	degenerated	-
$A_{1.2}$	1	1	2	3	degenerated
$A_{2.2}$	1	1	2	3	degenerated
$B_{5.2}$	1	1	2	3	degenerated
$C_{3.2}$	1	1	2	2	degenerated
$C_{4.2}$	1	1	2	3	degenerated
$C_{5.2}$	1	1	2	2	degenerated
$A_{1.3}$	1	1	2	2	degenerated
$B_{4.3}$	1	1	2	3	degenerated

Grade: 1,Single or several shining cells present throughout the well; 2, shining cells in small clumps of 1 to 5; 3, shining cell clumps in bigger size; 4, complete sheet formation.

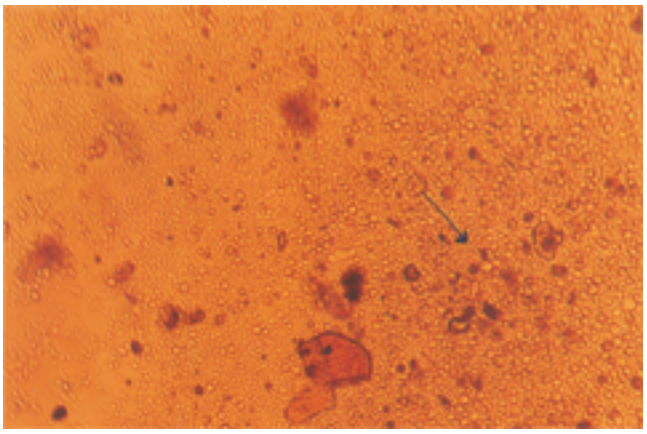

Fig 1. $B_{6.1}$ Clone

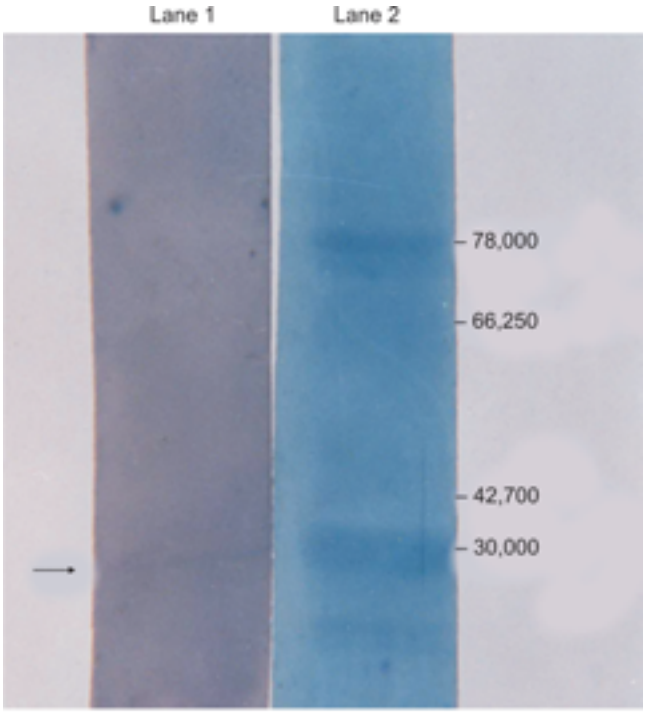

Fig. 2. Immunoblot of BHV-1.

Lane 1, Purified virus protein; Lane 2, carbonic anhydrase (30,000), ovalbumin (42,700), albumin (66,250), ovatransferrin (76–78,000).

the presence of cells. In 10^{-1} dilution single cells were found to be scattered throughout the plate. In 10^{-4} to 10^{-6} dilution about 10 to 20 single shining cells were found scattered throughout the plate. On third day, the wells which received 10^{-5} dilution of cells, one shining focus having 5–6 cells was observed and by eighth day after distribution, the focus was grown into a single clump as shown in Fig. 1. $A_{2.1}$ and $C_{1.1}$ on day 20th post fusion showed complete sheet formation whereas others appeared as small clumps of 1 to 5. Hybrid no. $A_{2.1}$ and $C_{1.1}$ degenerated on day 23rd post fusion. On day 28th of post-fusion all the clones degenerated. This may be due to the use of BALB/C mice which were not extensively

Table 3. Results of screening of hybrids for anti-BHV-1 antibodies

Hybrid no.	Days, post-fusion		
	13	15	23
$A_{2.1}$	-	1.24	-
$B_{6.1}$	-	1.37	3.54
$C_{1.1}$	-	1.70	-
$A_{1.2}$	-	1.33	2.00
$A_{2.2}$	-	1.85	2.50
$B_{5.2}$	-	1.22	-
$C_{3.2}$	-	1.33	2.00
$C_{4.2}$	-	1.55	2.00
$C_{5.2}$	-	1.15	-
$A_{1.3}$	-	1.14	2.20
$B_{4.3}$	-	1.15	2.30

-,indicates negative results; figures depict positive/negative ratio (P/N); A P/N ratio of 2.00 and above was taken as positive.

inbred and improper matching of major histocompatibility complex (MHC) which lead to high rate of chromosome loss and phenotypic instability (Srikumaran *et al.* 1990) and ultimately, degeneration.

Carter *et al.* (1986) recommended 10-14 days post-fusion as the right stage for starting the screening of the hybrids for antibody production. Out of the 11 clones observed, 7 clones showed positive reaction by ELISA (Table 3). Thus percentage of clones showing positive reaction in ELISA is 63.6%. Out of 24 hybrid clones produced by Kida *et al.* (1982), 12 clones (50%) secreted antibodies neutralizing BHV-1. The P/N ratio for the 7 clones is equal to or just above the cut off value (Table 3) and $B_{6.1}$ was having the highest P/N ratio of 3.54. Thus low level of antibodies was secreted and some hybrids were even negative for any antibodies, although sufficient growth was observed.

Since, $B_{6.1}$ clone was having highest P/N ratio, the supernatant from this clone was subjected to isotyping and it revealed only IgM immunoglobulin and OD value was 0.211 whereas OD of negative control was 0.012. Mohanty *et al.* (2000) produced monoclonal antibodies against New castle disease virus, some of which belonged to IgM class.

When the culture supernatant of clone $B_{6.1}$ was reacted with the blotted protein, the antibody recognized a single band of molecular weight 26–27 KD (Fig. 2). Sreenivasa *et al.* (1998) analyzed the immunoreactive polypeptides of herpesvirus by immunoprecipitation of radioactively labeled viral preparations. They obtained 13 polypeptides with molecular weight ranging from 25.6 to 141.3 KD.

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