Development of monoclonal antibodies against group A animal rotaviruses

B R Gulati *, R Pandey 1 and B K Singh
National Research Centre on Equines, Sirsa Road, Hisar 125 001, India
1Department of Veterinary Microbiology, CCS Haryana Agricultural University, Hisar 125 004, India

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Hybridomas secreting monoclonal antibodies (MAbs) to a group A rotavirus (GAR) were developed by fusion of myeloma cell (SP2/0) with spleen cells of BALB/c mice immunized with semi-purified bovine rotavirus (CR129, G10P11). Two of these rotavirus-specific monoclonal antibodies were further characterized by immunoblotting and also tested for their reactivity with rotaviruses of bovine, equine and porcine origin in a sandwich ELISA. MAb 6C7F7 (IgM isotype) showed reactivity with two proteins i.e. VP2 (95 kDa) and VP6 (44 kDa), while MAb 2H7E8 (IgG2b isotype) reacted with only 44 kDa VP6 protein of purified bovine rotavirus CR129 on immunoblotting. These VP6-specific MAbs were used as capture antibody in sandwich ELISA for testing with different group A rotavirus antigens. Both MAbs reacted equally with all the bovine and equine rotavirus isolates tested and also with two known rotavirus positive porcine stool samples. These findings suggest that one of these MAbs is directed specifically against a group-specific protein VP6 and will be useful to detect group A rotaviruses directly from stool samples in different animal species employing a sandwich ELISA.

Keywords: rotavirus, monoclonal antibody, bovine, equine, porcine, ELISA
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Introduction

Rotavirus has been recognized as a predominant cause of acute diarrhoea in infants of several animal species including humans1,2. Rotavirus, a member of family Reoviridae, consists of 11 segments of double stranded RNA (dsRNA) and a triple layered protein shell comprising six structural proteins (VP1, VP2, VP3, VP4, VP6 and VP7). The inner most protein layer consists of 120 VP2 molecules enclosing a few copies of VP1 and VP3 proteins and the viral genome. The middle protein layer, also termed inner capsid, is composed of 780 molecules of VP6, the most abundant virion protein. The outermost protein layer (outer capsid) is composed of 120 molecules of VP4 and 780 molecules of glycoprotein, VP7. Antigenically, rotaviruses are classified into 7 groups (A through G) based on antigenic differences on the inner capsid protein (VP6). Most epidemiologically significant rotaviruses of man and animals belong to group A3. VP6 also possesses subgroup specificity; based on which group A rotaviruses have been classified into subgroups. Subgroup I is commonly associated with animal rotaviruses, while subgroup II with human strains. The two outer capsid proteins, VP4 and VP7, are involved in neutralization of viral infectivity. VP7 is the major neutralizing antigen and VP4 being the minor one2,4. Based on antigenic differences in these outer capsid proteins, group A rotaviruses have been classified into at least 20 different P (VP4) types and 14 G (VP7) types2,5.

Rapid and accurate detection of rotaviruses is important to prevent the spread of infection. Enzyme-linked immunosorbent assay (ELISA) is the most widely used method for the diagnosis of rotavirus infection in stool specimens. Other diagnostic techniques, viz, electron microscopy and polyacrylamide gel electrophoresis followed by silver staining of RNA (RNA-PAGE) are employed but require more efforts and expertise than ELISA. However, ELISA with polyclonal antisera has the disadvantage of often giving false-positive results6,7. On the other hand, sandwich ELISA employing combination of polyclonal and monoclonal antibodies (MAbs) is more efficient for the detection of rotavirus from stool samples8. MAbs directed against the common group antigen (VP6) of rotaviruses of human and different animal species have been developed abroad, but these are not available indigenously. In the present study, MAbs against group A bovine rotavirus were developed and characterized for their

*Author for correspondence:
Tel: 91-1662-276151, 276748, 275114 Ext. 211
Fax: 91-1662-276217
E-mail: baldevgulati@sancharnet.in
application in immunoassays for the detection of rotavirus in different animal species.

Materials and Methods

Viruses and Cells

Bovine rotavirus strains UK, NCDV and B223 were kindly supplied by Dr Campbell, Moredun Research Institute, Edinburgh (UK) while CR129 and CR156 were indigenous isolates from diarrhoeic calves. Equine rotaviruses (FRV2, FRV3, FRV25 and FRV28) were isolated from diarrhoeic foals in and around Hisar. In addition, two group A rotavirus-positive stool samples (by RNA-PAGE) collected from diarrhoeic pigs of a farm around Hisar and a fecal sample containing non-group A bovine rotavirus were included in the study.

The cell culture-adapted rotaviruses were grown in MA104 cells in the presence of trypsin, as previously described. The cell culture supernatant was clarified by centrifugation at 12,000 G for 15 min, followed by pelleting in Beckman ultracentrifuge at 100,000 G for 2 h. The pellet was dissolved in 1/50th volume of 0.05 M Tris-HCl, pH 7.5 containing 150 mM NaCl and 10 mM CaCl₂ (TNC) buffer and purified by rate zonal sucrose density gradient centrifugation and the rotavirus-specific band was dissolved in TNC and again pelleted. The final pellet was dissolved in TNC buffer and stored at −20°C till further use. The myeloma cells SP2/0 used for fusion were grown in RPMI-1640 medium supplemented with 20% fetal calf serum.

RNA-PAGE

Cell culture supernatant and fecal samples collected from diarrhoeic animals were screened for the presence of rotavirus by polyacrylamide gel electrophoresis followed by silver staining.

MAb Production

BALB/c mice 8-10 week old were immunized with 100 μg of semi-purified bovine rotavirus CR129, adjuvanted with Freund complete adjuvant. This was followed 3 weeks later by immunization with the same virus adjuvanted with Freund incomplete adjuvant. Immunizations continued until the enzyme-linked immunosorbent assay (ELISA) Three in serum reached between 32000 and 64000. days prior to fusion, the mice were inoculated with non-adjuvanted virus intraperitoneally. The spleen cells (10⁶ to 10⁷) were isolated and mixed with SP2/0 myeloma cells in the ratio of 10:1. After centrifugation at 100 × g, 1 mL of 40% polyethylene glycol was layered onto the cell pellet, gently stirred and incubated at 37°C for 1 min. Then 10 mL of selective hybridoma medium (RPMI-1640 supplemented with 20% fetal bovine serum and hypoxanthine-aminopterin-thymidine) was added dropwise and then the suspension made up to 50 mL with hybridoma medium. The cells were dispensed in five 96-well tissue culture plates containing feeder mouse peritoneal macrophages, prepared one-day prior to fusion.

All the hybridoma culture supernatants were screened against homologous antigen by both ELISA and immunofluorescence (IF). The hybridoma cultures secreting antibodies against rotavirus were cloned at least two times using limiting dilutions technique. MAbs as ascites were raised by growing antibody secreting clones in syngeneic mice primed with pristane (Sigma).

Screening ELISA

For screening hybrids, the sandwich ELISA was performed following the method of Green et al. Briefly, ELISA plates (Nunc) were coated with optimal dilution of guinea pig anti-rotavirus hyperimmune serum (a gift from Dr Y Matsuda, Akita University School of Medicine, Japan) followed by addition of CR129-infected tissue culture supernatant. Hybrid culture fluids were tested at dilutions of 1:2 and 1:5 in phosphate buffered saline (PBS, pH 7.2). Bound antibody was detected with rabbit anti-mouse IgG conjugated to horseradish peroxidase (Sigma). Optimum dilutions of reagents were determined by checkerboard titration.

Isotyping

For identification of the MAb isotype, all the MAbs were tested by ELISA with goat isotype-specific antisera (Isotyping kit ISP2, Sigma) as per manufacturer’s recommendation.

IF

Hybrid culture fluids were added to CR129-infected MA104 cells grown in 96-well plates and antibody binding was detected after staining with goat anti-mouse IgG conjugated with fluorescein isothiocyanate (Sigma), as per the method of Coulson et al.

Immunoblotting

Purified rotavirus proteins (CR129) were denatured by boiling under reducing conditions and proteins
separated in 11% PAGE gel in discontinuous buffer system and transferred on nitrocellulose membrane. Molecular weight standards (6H and VII-L, Sigma) were run simultaneously in the gel to calculate the molecular weights of separated rotavirus proteins. MAbs with optimal dilutions were incubated for 1 h at 37°C with rotavirus (CR129) proteins transferred on nitrocellulose. The reactivity of MAb with rotavirus proteins was detected with rabbit anti-mouse IgG conjugated to horseradish peroxidase.

Cross-reactivity of MAb with Different Rotavirus Antigens

Cross-reactivity of MAbs with rotavirus antigens (tissue culture supernatant or 10% faecal sample in PBS) of different animal origin was tested in duplicate wells. The rotavirus antigen was added to ELISA plates coated with rabbit anti-rotavirus hyperimmune serum incubated overnight at 4°C. This was followed by capture of antigens by adding MAb for 1 h at 37°C. Subsequent steps were similar to those of sandwich ELISA used for screening hybrids.

Results and Discussion

Production of MAb

On fusion of rotavirus-immunized mouse splenocytes with mouse myeloma cells, 12 hybrids reacted to rotavirus CR129 by ELISA, giving OD492 value above 0.2 at 1:5 dilution of hybridoma supernatants (1.5 times mean negative control OD492). All the hybrids were also screened by IF and 12 hybrids (same as in ELISA) were secreting rotavirus-specific antibodies. It has been previously shown that screening of hybridoma culture supernatant for rotavirus antibodies by ELISA led to preferential selection of hybridomas directed against the inner capsid protein, VP613,14. However, Zheng et al16 reported that the ELISA was less sensitive than immunofluorescence assay for screening of MAbs, other than those reacting with the VP6. Hence, IF was also included for screening of hybrids. Contrary to previous findings, there was no difference in the number of hybrids detected for rotavirus-specific antibodies by the two screening assays.

Two of the hybrids (2H7E8 and 6C7F7) that were strong positive for rotavirus-specific antibodies (OD492 above 0.6 in ELISA and strong positive in IF) were cloned twice by limiting dilution method, amplified and ascites raised in BALB/c mice. The ELISA titre of MAbs 2H7E8 and 6C7F7 in ascites fluid were 5120 and 2560, respectively. Isotyping revealed that MAb 2H7E8 was IgG2b while MAb 6C7F7 belonged to IgM isotype.

Polypeptide Specificity of MAb to Rotavirus Proteins

The rotavirus proteins of semi-purified CR129 were separated by SDS-PAGE and transferred onto nitrocellulose membrane to establish the polypeptide specificity of both MAbs. MAb 2H7E8 bound to a protein of 44 kDa corresponding to VP6, a group specific inner capsid protein of rotavirus (Fig. 1). MAb 6C7F7, on the other hand, bound to two proteins of 95 kDa and 44 kDa, corresponding to VP2 (viral core) and VP6 (inner capsid) proteins, respectively (Fig. 1). These proteins were also detected in the Western blots using polyclonal guinea pig hyperimmune serum to rotavirus O510.

MAbs directed against major inner capsid protein VP6 of rotaviruses from bovine, porcine, equine, avian and human origin have been developed by various workers13,14,16-20. This is the first report of development of MAbs against group A animal rotavirus from India. VP6 is the most abundant protein of rotavirus particle (55% of total virion protein) and highly antigenic. Therefore, it has been observed that even if mice are immunized with whole virion, majority of antibodies (95%) are directed against this protein13,14,21.

MAb 6C7F7 reacted with two different polypeptide species. The possibility of mixed population of cells in this clone was ruled out by re-cloning one more time. This might be due to the fact that different structural proteins of rotaviruses have group specific epitopes and there is a possibility that some of these antigenic epitopes are conserved among different proteins of rotaviruses and hence recognized by the same monoclonal antibody. Similar observations of one MAb reacting with several polypeptides of rotaviruses have been reported earlier13,15,21. It may be considered that various antigenic determinants are not necessarily localized on a single polypeptide and may be shared between different polypeptides and, on the other hand, one polypeptide generally carries several different antigenic determinants.

Cross-reactivity of MAb to Rotavirus of Different Serotypes of Animal Species

The cross-reactivity of MAbs was tested in sandwich ELISA to a series of bovine rotavirus strains of different serotypes, equine rotavirus isolates and known rotavirus-positive stool samples of porcine origin. The results are presented in Table 1. MAb 2H7E8 reacted equally in ELISA with all group A rotavirus strains of bovine and equine origin and also with rotavirus-positive stool samples of porcine origin. The cross-reactivity of MAb 6C7F7 was lower than that of MAb 2H7E8.
origin. Similarly, MAb 6C7F7 reacted with all the strains tested (Table 1). Both these MAbs were tested in ELISA with a fecal antigen of non-group A bovine rotavirus and none of the MAbs reacted with this sample (ELISA OD492 being less than 0.15).

There are 14 known G (VP7) types and about 20 P (VP4) types of group A rotaviruses in animals and humans. Not all types are reported from each host species and only a few serotypes are commonly reported from each animal species and humans. Monoclonal antibody directed against a group-specific protein should react equally with all the serotypes within a group. The findings of the present study indicate that the two MAbs described here are not serotype-specific, since these reacted equally with different group A rotaviruses i.e. bovine rotaviruses of different serotypes, and also with equine and porcine rotaviruses. On the other hand, the MAbs did not react with a non-group A rotavirus tested.

These MAbs are directed against the common group antigen, VP6, reacting equally with all the group A rotaviruses of different serotypes and from different species of animals. Hence MAbs secreted by these clones seem to be directed against group antigen, identifying all group A rotaviruses from different animal origin. VP6 also possesses subgroup specificity; based on which group A rotaviruses have been classified into subgroups. Subgroup I is commonly associated with animal rotaviruses, while subgroup II with human strains. Bovine reference strains (UK, NCDV, B223) used in the present study belong to subgroup I specificity. No known rotavirus of subgroup II specificity was tested with these MAbs. Subgroup specificity of these MAbs needs to be ascertained using some human group A rotaviruses of subgroup II, as most of animal rotaviruses belong to subgroup I.

**References**


**Table 1—Reactivity of rotavirus-specific MAbs with different rotaviruses in ELISA**

<table>
<thead>
<tr>
<th>Rotavirus strain</th>
<th>G and P type</th>
<th>ELISA Mean OD492 (±S.D) value with MAb*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>2H7E8</td>
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<tr>
<td>Bovine rotavirus strains</td>
<td></td>
<td></td>
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<tr>
<td>CR129</td>
<td>G10P11</td>
<td>1.104 (±0.15) 1.150 (±0.1)</td>
</tr>
<tr>
<td>CR156</td>
<td>G10P11</td>
<td>1.51 (±0.04) 1.339 (±0.04)</td>
</tr>
<tr>
<td>UK</td>
<td>G6P5</td>
<td>0.892 (±0.02) 0.931 (±0.02)</td>
</tr>
<tr>
<td>NCDV</td>
<td>G6P1</td>
<td>0.541 (±0.01) 0.611 (±0.02)</td>
</tr>
<tr>
<td>B223</td>
<td>G10P11</td>
<td>0.699 (±0.01) 0.808 (±0.01)</td>
</tr>
<tr>
<td>Equine rotavirus isolates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRV2</td>
<td>NT</td>
<td>1.216 (±0.00) 1.086 (±0.02)</td>
</tr>
<tr>
<td>FRV3</td>
<td>NT</td>
<td>1.173 (±0.00) 1.092 (±0.02)</td>
</tr>
<tr>
<td>FRV25</td>
<td>NT</td>
<td>1.589 (±0.02) 1.322 (±0.01)</td>
</tr>
<tr>
<td>FRV28</td>
<td>NT</td>
<td>1.179 (±0.03) 1.243 (±0.03)</td>
</tr>
<tr>
<td>Porcine rotavirus-positive stools</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P316</td>
<td>NT</td>
<td>0.516 (±0.00) 0.498 (±0.00)</td>
</tr>
<tr>
<td>P317</td>
<td>NT</td>
<td>0.718 (±0.00) 0.704 (±0.01)</td>
</tr>
<tr>
<td>Uninfected cell control</td>
<td></td>
<td>0.097 (±0.01) 0.192 (±0.00)</td>
</tr>
</tbody>
</table>

*Culture supernatant of a hybridoma known negative for rotavirus antibody (negative control MAb) gave mean OD492 value below 0.15.*
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