Isolation and Characterization of Anti-Rotavirus Immunoglobulins Secreted by Cloned Hybridoma Cell Lines

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SUMMARY

Five monoclonal hybridoma cell lines secreting antibodies against bovine rotavirus have been produced and four of them characterized by immunostaining of structural polypeptides electrophoretically transferred on to nitrocellulose sheets. Three hybridomas appeared to be directed against the major structural polypeptide (VP39) of the virion. These three monoclonals cross-reacted with the major polypeptide of simian rotavirus and human rotavirus. A fourth hybridoma appeared to react specifically with the high-molecular weight external polypeptide (VP89) and its cleavage products. A cross-reaction was observed with human Wa strain but not with SA11. The fifth hybridoma, even though reacting in an immunofluorescent test, did not show any reactivity by immunostaining. None of the monoclonals neutralized the infectivity of bovine rotavirus.

Rotaviruses have emerged during the last decade as important aetiological agents of enteric diseases in man and animals (McNulty, 1978; Flewett & Woode, 1978). The biology of these viruses is far from being fully understood. Furthermore, the antigenic relationships between viruses originating from different species have not yet been clearly established, nor is it known whether interspecies infections occur naturally. Obviously, there is a need for immunological markers, precise enough to identify correctly the most representative virus types which exist in nature and to facilitate the study of other aspects of virus biology. In this context, there is little doubt that the application of the somatic cell hybrid technique introduced by Köhler & Milstein (1975) and the isolation of monoclonal antibodies, would be useful in different fundamental and applied areas. The present work was aimed at establishing such somatic cell hybrids secreting monospecific anti-rotavirus antibodies.

Bovine rotavirus (Thiverval strain) (L'Haridon & Scherrer, 1976) was grown in MA 104 cells in Eagle's basal medium (MBE 0011, Eurobio, France) supplemented with trypsin (0.0017 U/ml), penicillin (100 IU/ml) and streptomycin (0.1 mg/ml). Rotavirus D and L particles were extracted and purified as described previously (Cohen, 1977). The purified preparations were used to immunize eight Balb/c mice 2 months old and serologically negative against rotavirus. Each animal was injected intraperitoneally with 60 μg purified virus (D + L particles) in 0.1 ml saline with 0.1 ml complete Freund's adjuvant. Two weeks following the priming dose, all mice had responded and the two with the highest anti-rotavirus titres were given a booster injection. The mice were sacrificed 3 days later. Their spleen cells were mixed with immunoglobulin-non-secreting, 8-azaguanine-resistant SP2-0 myeloma cells (Shulman et al., 1978) and fused with 50% polyethylene glycol, following the technique described by Galfrè et al. (1977). Fused cells were selected in a hypoxanthine/aminopterin/thymidine (HAT) selective medium.

The first screening of cell culture supernatants was performed by indirect immunofluorescence (IIF) 15 days after fusion. The antigen consisted of MA 104 cells in Eagle's basal medium (MBE 0011, Eurobio, France) supplemented with trypsin (0.0017 U/ml), penicillin (100 IU/ml) and streptomycin (0.1 mg/ml). Rotavirus D and L particles were extracted and purified as described previously (Cohen, 1977). The purified preparations were used to immunize eight Balb/c mice 2 months old and serologically negative against rotavirus. Each animal was injected intraperitoneally with 60 μg purified virus (D + L particles) in 0.1 ml saline with 0.1 ml complete Freund's adjuvant. Two weeks following the priming dose, all mice had responded and the two with the highest anti-rotavirus titres were given a booster injection. The mice were sacrificed 3 days later. Their spleen cells were mixed with immunoglobulin-non-secreting, 8-azaguanine-resistant SP2-0 myeloma cells (Shulman et al., 1978) and fused with 50% polyethylene glycol, following the technique described by Galfrè et al. (1977). Fused cells were selected in a hypoxanthine/aminopterin/thymidine (HAT) selective medium.

The first screening of cell culture supernatants was performed by indirect immunofluorescence (IIF) 15 days after fusion. The antigen consisted of MA 104 cells infected with bovine rotavirus. Uninfected cells were used as controls. Cells were cultured in test plastic plates (Falcon microtest II, Tissue culture 3042), fixed with alcohol 24 h after infection and stored at −80 °C until use.

Positive hybridoma cultures were cloned in 96-well microplates using the limiting dilution procedure and mink Mv1Lu (NBL-7) cells (American Type Culture Collection) as feeder cells. After 10 to 15 days, the supernatants of growing clones were rescreened by IIF and subcultures stored in liquid nitrogen in foetal calf serum containing 10% dimethyl sulphoxide.

Key words: hybridomas/monoclonal antibodies/rotaviruses/immunostaining of proteins
Fig. 1. Immunostaining of bovine rotavirus structural polypeptides and cross-reactivity of monoclonals 160C19, 86C20 and 188E22 with human and simian rotavirus polypeptides. The structural polypeptides of bovine rotavirus were separated by polyacrylamide gel electrophoresis and subsequently transferred on to nitrocellulose filters. Test strips were allowed to react with an appropriate dilution (1:30) of the different hybridoma culture supernatants according to Towbin et al. (1979). A positive control was run with hyperimmune mouse serum diluted 1:270. The bands that can be seen after Coomassie Brilliant Blue staining are indicated by arrows. The black line at the top of the strips corresponds to the top of the gel. Human and simian rotavirus polypeptides were prepared from virus grown in MA104 cells. Bovine rotavirus was produced in experimentally infected calves. All strips were prepared from gels run with identical amounts of proteins. TR, Bovine rotavirus (Thiverval strain); Wa, human rotavirus type 2; SA11, simian rotavirus.

Out of a total of 144 cultures which were screened for the presence of anti-rotavirus antibodies, 19 were found to be positive and subsequently subjected to cloning. In a proportion of these positive cultures detectable antibody activity eventually disappeared but five of the cultures gave rise to established lines which were maintained in culture for up to 10 months and which proved stable in secreting antibodies. Larger quantities of antibodies were obtained by intraperitoneal injection of $2 \times 10^6$ hybridoma cells into pristane-treated Balb/c mice. All these mice produced ascitis within 7 to 14 days and about 15 ml ascitic fluid was obtained from each mouse.

All hybridoma cell clones secreted substantial amounts of antibodies as revealed by IIF testing of supernatants: two secreted antibodies of the IgG1 class (160C19 and 188E22) and three of the IgG2a subclass (86C20, 92A15 and 159S17) as determined by the immunodiffusion method. The highest IIF titres were repeatedly recorded with hybridoma lines 92A15 (1/6016), 159S17 (1/6016) and 188E22 (1/1504) and the lowest with line 160C19 and 86C20 (1/16). None of the five hybridomas secreted antibodies with neutralizing activity.

When the cell supernatants were reacted with purified virion polypeptides in the immunostaining test described by Towbin et al. (1979) four hybridomas were shown to react. Despite positive IIF tests no reactivity could be demonstrated by immunostaining using 92A15 hybridoma supernatants even working with undiluted samples. It is possible that the corresponding antigenic sites have been denatured during SDS-PAGE.

As shown in Fig. 1, clonal antibodies from hybridoma 159S17 and 160C19 reacted exclusively with VP39 which is the major structural protein of bovine rotavirus (Cohen et al., 1979; Novo & Espanza, 1981).

Antibodies from hybridoma 86C20 reacted with VP39 also but another band corresponding to a polypeptide of about 20 kdal which is usually not detectable by Coomassie Brilliant Blue is also revealed by immunostaining, suggesting that these two polypeptides are antigenically related. Alternatively, the 86C20 population may not be truly monoclonal.
Clonal antibodies from hybridoma 188E22 permitted the detection of several bands. The most heavily stained bands were localized in the 89 kdal and the 62 kdal regions; other faint bands that were not seen by Coomassie Brilliant Blue staining were also apparent. These results suggested either that the 188E22 preparation was a mixture of distinct hybridomas or that it reacted with a structural protein of high molecular weight and its cleavage products. In that case the E22 population was carefully recloned and the new clones tested again by immunostaining. Identical results were obtained; thus, it seems reasonable to assume that the 188E22 monoclonal antibodies react with one of the high-molecular weight polypeptides (VP3 according to Estes et al., 1981 and Espejo et al., 1981) corresponding to our VP89 which is known to be cleaved into polypeptides of 60 and 28 kdal by proteolytic enzymes.

Additional studies were aimed at comparing the reactivity of the hybridoma preparations with other rotaviruses, namely the human Wa strain (Wyatt et al., 1980) and the simian rotavirus (SA11). As shown in Fig. 1 monoclonal antibodies from hybridoma 160C19 and 86C20 are not species-specific as they reacted almost equally well with the major structural protein of bovine, human and simian rotaviruses. This agrees with earlier findings, indicating that VP39 carries the group antigen(s) of rotaviruses. Whether or not these monoclonals are specific for the same epitope is not yet known. Monoclonals of 188E22 cell line also cross-reacted with the high-molecular weight polypeptide extracted from human virus but to a lower degree as compared with bovine rotavirus; furthermore, no cleavage products were detected with human virus. With simian rotavirus no reaction at all could be demonstrated, suggesting that an antigenic difference may well exist at this level between bovine or human and simian rotaviruses.

Attempts to demonstrate a cross-reactivity with S17 monoclonals were not successful. It is possible that these antibodies recognize a determinant which may not be present in human Wa strain or in SA11 rotavirus.

In spite of the lack of hybridomas directed against type-specific neutralizing antigens, those which are already available will be useful especially in work aimed at improving the diagnostic procedures of rotavirus infections and in comparing the antigenic properties of different isolates.

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