Inhibition of rotavirus in vitro transcription by optimal concentrations of monoclonal antibodies specific for rotavirus VP6

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Three monoclonal antibodies (MAbs) obtained from inoculation of mice with either a serotype 1 human rotavirus or rotavirus SA11 (serotype 3) inhibited the in vitro transcription of rotavirus SA11. Two of the MAbs exhibited a biphasic inhibitory response. Removal of antibody from MAb preparations by adsorption with Sepharose-Protein G reduced the inhibitory activity completely for all three MAb preparations. Analysis by radioimmunoprecipitation and Western blotting indicated that all three MAbs reacted with VP6. All MAbs also reacted with four group A rotavirus serotypes by ELISA, but did not cross-react with reovirus type 1, poliovirus type 2 or MA-104 cell lysates. Transcription of four rotavirus serotypes as well as epizootic diarrhoea of infant mice rotavirus was inhibited when tested with two of the MAbs. Transcription of both purified single-shelled virus and purified heat-activated double-shelled SA11 rotavirus was inhibited by purified MAb. Our results indicate that these MAbs can be used effectively to study the events associated with rotavirus transcription.

Single-shelled rotavirus particles possess a fully functional RNA-dependent RNA polymerase (Cohen, 1977). Intact single-shelled particles, intact heat-activated double-shelled particles (60 °C for 30 s) or EDTA-treated double-shelled particles have RNA transcriptase activity (Cohen, 1977; Spencer & Arias, 1981). Attempts to demonstrate RNA polymerase activity with purified viral proteins (VPs) have been unsuccessful (Bican et al., 1982). However, sequence information suggests that VP1 is the RNA polymerase (Cohen et al., 1989), and photoaffinity labelling studies with 8-azido-ATP have confirmed this (Valenzuela et al., 1991).

VP2 has been shown to bind ssRNA, dsRNA and dsDNA, suggesting possible involvement in transcription activity (Boyle & Holmes, 1986; Kumar et al., 1989; Labbe et al., 1991). However, there has been no demonstration that VP2 actively participates in the synthesis of mRNA. Recently, studies have shown that VP3 possesses guanylyltransferase activity (Pizarro et al., 1991). A relationship between VP6 and RNA transcriptase activity has been suggested by evidence that the reassociation of purified VP6 with core particles results in recovery of RNA transcription activity (Bican et al., 1982; Sandino et al., 1986). These studies, however, did not clarify the role of VP6 in this enzymatic process. Reassociation of VP6 with the core particle may have provided the proper conformation for other proteins within the single-shelled particle to synthesize mRNA. A domain essential for the assembly of VP6 with single-shelled particles has been mapped to the carboxyl end of VP6 and a domain for trimerization resides near the centre of VP6 (Clapp & Patton, 1991).

The lack of suitable reagents to examine rotaviral enzymatic activity has hindered research on this issue. The RNA-dependent RNA polymerase of rotavirus particles is insensitive to the effects of the eukaryotic RNA polymerase inhibitors rifampicin, actinomycin D and α-amanitin (Spencer & Arias, 1981). Similarly, no polyclonal or monoclonal antibodies (MAbs) have been shown to inhibit enzyme activity in intact single-shelled particles. This manuscript documents MAbs specific for a rotavirus structural protein (VP6) that inhibit rotavirus RNA-dependent RNA polymerase activity.

Simian rotavirus SA11, human rotaviruses (strains Wa, S2, YO and ST 3) and epizootic diarrhoea of infant mice (EDIM) rotavirus strain W were prepared in MA-104 cells. For propagation, rotaviruses were activated with Enzar-T trypsin (Armour Pharmaceutical) at a concentration of 20 units/ml for 30 min at 37 °C. Viruses were purified by freon extraction and CsCl isopycnic ultracentrifugation using methods similar to those reported previously (Hughes et al., 1973).

MAbs were produced using a procedure described by Oi & Herzenberg (1980) using Robertsonian mice inoculated intraperitoneally with CsCl-purified single-shelled or double-shelled SA11 rotavirus. Hybridoma
cells 2A11/E9 and 8H2/G5 were obtained in essentially the same manner after inoculation of BALB/c mice with a purified preparation of a culture-adapted serotype 1 human rotavirus.

Mouse MAb s were purified from ascites fluid by Sepharose–Protein G affinity chromatography. Ascites fluid (5 ml) was dialysed twice against 150 ml 20 mM-sodium phosphate buffer (buffer A). Samples were then loaded at 1.0 ml/min onto a column containing 6 ml Sepharose–Protein G4 Fast Flow gel (Pharmacia) with bed dimensions of 1.4 × 3.7 cm. Additional buffer A (40 ml) was pumped through the column to remove the unbound sample material, then 15 ml 20 mM-sodium citrate pH 5-0 (buffer B) was pumped through the column at 1.0 ml/min. Next, a pH gradient from pH 5-0 to 2-5 was generated with a gradient maker using 30 ml 60 mM-glycine pH 2-2 (buffer C) and 30 ml buffer B. Purified IgG was eluted between pH 3-2 and 2-6. To prevent inactivation of acid-labile IgG, 100 Ixl 1 M-Tris base pH 8-0 per ml of eluted material was added to each tube collecting the fractions containing the purified IgG. MAb s were also partially purified from ascites fluid by QAE ion-exchange chromatography. Ion-exchange chromatography discs (Cuno Laboratories) were employed using a procedure developed by the makers.

Antibodies specific for simian rotavirus SA11 and human rotaviruses were detected using a solid-phase ELISA developed by Hawkes et al. (1982). The class and subclass of each MAb were determined using a solid-phase ELISA and an isotyping kit for murine MAb s (Screen Type, Boehringer Mannheim). The antibody class and subclasses were IgG2b for MAb s 2A11/E9 and 8H2/G5, and IgG2a for MAb B22/9.

For in vitro transcription assays, CsCl-purified single-shelled or double-shelled rotavirus was used. Virus, at a concentration of 0-06 mg/ml, was mixed with antibody diluted in 100 mM-Tris–HCl pH 8-0, and incubated at 4 °C overnight. Double-shelled rotavirus was heat-activated at 60 °C for 2 min before being used in a transcription assay. Ten μl of the virus–antibody sample was added to 45 μl of a transcription mixture composed of the following reagents dissolved in 100 mM-Tris–HCl pH 8-0, 2-5 μl each of ATP, CTP and GTP, 0-625 mM-UTP, 0-5 mM-S-adenosylmethionine, 8-0 mM-phosphoenolpyruvate, 12 mM-MgCl₂, 50 μg/ml pyruvate kinase, 0-2% bentonite and 5 μCi [³H]UTP (Flores et al., 1982). This mixture was incubated at 37 °C for 2 h. Acid-insoluble material was precipitated with cold 5% trichloroacetic acid and collected onto nitrocellulose filters. Filters were dried and radioactivity was quantified by liquid scintillation counting using a Beckman LS 7000 scintillation counter.

Viral proteins for radioimmunoprecipitation assays (RIPAs) were pulse-labelled with [³S]methionine and RIPAs were performed using the method of Greenberg et al. (1983). In order to minimize non-specific binding of proteins to the Staphylococcus aureus cells, these cells were preincubated (blocked) with non-radiolabelled uninfected cell lysate. At the conclusion of the RIPA, samples for PAGE were transferred to clean siliconized microfuge tubes, heated in a boiling water bath for 5 min and centrifuged at 15 600 g for 1 min to remove bacterial cells.

For Western blotting, electrophoresed viral proteins were transferred electrophoretically to nitrocellulose membranes (Bio-Rad, 0-45 μm) at 30 V overnight (Towbin et al., 1979). Electroblotted proteins were detected with an India ink stain (Hughes et al., 1988) and by using a solid-phase ELISA developed by Hawkes et al. (1982).

Affinity-purified MAb s were tested for their effect on SA11 rotavirus transcription. Transcription was inhibited between 95 and 100% by MAb 2A11/E9 (P < 0-01), when compared to transcription levels produced by virus alone (Fig. 1a). Inhibition of transcription occurred at antibody concentrations ranging from 13 μg/ml to 13-5 mg/ml. Both MAb s 8H2/G5 and B22/9 exhibited a biphasic inhibitory response (Fig. 1b). Antibody
8H2/G5 inhibited transcription between 59 and 96% \((P < 0.01)\) at antibody concentrations ranging from 0.8 to 844 \(\mu\)g/ml. Transcription was inhibited between 76 and 99% \((P < 0.01)\) for MAb B22/9 at concentrations of 3 \(\mu\)g/ml to 3.4 mg/ml. Analysis of the three affinity-purified MAb preparations by PAGE demonstrated that only homogeneous antibody was present. No other proteins were detected in the silver-stained gels (data not shown).

To provide additional evidence that the MAbs were responsible for inhibition of transcription, the MAbs were removed by adsorption with Sepharose–Protein G from purified preparations. MAbs, partially purified by ion-exchange chromatography, were treated with Sepharose–Protein G or Sepharose CL-4B; the latter was used as a control for non-specific binding of proteins to the Sepharose bead matrix. Sepharose CL-4B contains Sepharose beads with the same diameter and pore size as Sepharose–Protein G preparations, but the matrices do not have covalently linked Protein G molecules. MAb 2A11/E9 treated with Sepharose CL-4B inhibited SA11 virus transcription when compared to transcription levels produced by virus alone (Fig. 2a). The MAb inhibited transcription between 84 and 87% at antibody concentrations of 53 and 844 \(\mu\)g/ml, respectively. In comparison, Sepharose–Protein G-treated MAb 2A11/E9 did not inhibit transcription activity. Analysis by ELISA determined that 99.97% of the MAb had been removed (Fig. 2d). Removal of the MAb by Sepharose–Protein G adsorption reduced the sample’s inhibitory activity completely when compared to the Sepharose CL-4B control \((P < 0.01)\). Sepharose CL-4B-treated MAbs 8H2/G5 and B22/9 exhibited a biphasic response (Fig. 2b, c). Both MAbs inhibited transcription by greater than 70% at 53 \(\mu\)g/ml. In contrast, adsorption of the MAb 8H2/G5 sample with Sepharose–Protein G removed the inhibitors of transcription \((P < 0.01)\). ELISA demonstrated that 99.99% of the MAb was removed from the treated preparations. Removal of 98.4% of

Fig. 2. Effect of Sepharose–Protein G-adsorbed MAb samples on rotavirus transcription. Ion-exchange-purified MAbs (50 \(\mu\)l) at a concentration of 3.4 mg/ml were mixed with 50 \(\mu\)l 40% Sepharose–Protein G (○) or 40% Sepharose CL-4B (●) and incubated for 1 h at 37 °C. The Sepharose beads were pelleted by centrifugation and supernatants were treated again with an equal volume of the Sepharose preparations. The supernatants were then mixed with single-shelled SA11 virus and incubated at 4 °C overnight. Next, the samples were assayed for transcription activity with MAb 2A11/E9 (○), MAb 8H2/G5 (●) or MAb B22/9 (○). (d) ELISA titres of Sepharose CL-4B (●) or Sepharose–Protein G-treated (○) MAb preparations. Virus alone produced transcription levels of 2.9 pmol \(^{[3]}\)HUMP/\(\mu\)g virus (100% control). Standard error is displayed only for those values in which the error bars are wider than the data point symbol.
MAb B22/9 by adsorption completely eliminated the inhibitors from the sample \((P < 0.01)\). Sufficient quantities of the inhibitor were also removed by adsorption with single-shelled SA11 virus to abolish 100% of the inhibitory activity observed in partially purified preparations of MAb 2A11/E9 and 8H2/G5, and 50% in the MAb B22/9 preparation (data not shown).

The viral protein binding specificity of each MAb was determined by radioimmunoprecipitation and Western blotting using affinity-purified antibodies. All three MAbs immunoprecipitated VP2 and VP6. The intensity of these two protein bands decreased as the MAb concentration decreased. This suggested that the antibodies were specifically binding to these two viral proteins or a complex of these two proteins, or that single-shelled virus was being immune-precipitated. To resolve this problem, Western blotting analysis was used to clarify whether these MAbs were binding to a conformational epitope involving both VP2 and VP6, to similar epitopes on both VP2 and VP6 or to only one of these viral proteins. Affinity-purified MAb 2A11/E9, 8H2/G5 and B22/9 reacted only with VP6 from Western-blotted rotavirus proteins (data not shown). Normal mouse serum or ascites fluid containing a MAb reactive with rotavirus proteins (data not shown). Normal mouse serum or ascites fluid containing a MAb reactive with the CD4 molecule on lymphocytes did not react with blotted single-shelled rotavirus proteins.

MAbs 2A11/E9 and 8H2/G5 were tested for their effect on transcription of six different group A rotaviruses (Wa, S2, SA11, YO, ST 3 and EDIM). Transcription of all six rotaviruses was inhibited between 71 and 91% \((P < 0.01)\) by MAb 2A11/E9 when transcription levels of each virus-antibody mixture were compared to those levels produced by the same virus alone. Transcription of all rotavirus strains was also inhibited between 70 and 88% \((P < 0.01)\) by MAb 8H2/G5. This antibody demonstrated a biphasic inhibitory response with each of the six rotaviruses (data not shown).

Transcription of heat-activated double-shelled SA11 virus was also inhibited by both MAb to an extent comparable to that observed with single-shelled rotavirus (data not shown). MAb 2A11/E9 inhibited double-shelled rotavirus transcription between 94 and 98% \((P < 0.01)\), whereas MAb 8H2/G5 exhibited a biphasic response inhibiting transcription between 53 and 95% \((P < 0.01)\).

Transcription-inhibiting MAbS were also tested for ELISA reactivity against human rotavirus serotypes 1 to 4. For control purposes, the MAbS were reacted with reovirus type 1, poliovirus type 2 and MA-104 cell lysates. All three MAbS reacted strongly with the four rotavirus serotypes. No difference in reactivity occurred between viruses of different subgroups and no reactivity was observed with reovirus, poliovirus or the cell lysates.

A relationship between VP6 and RNA synthesis has been suggested by evidence that reassociation of purified VP6 with core particles results in recovery of RNA transcriptase activity (Bican et al., 1982; Sandino et al., 1986). This reassociation has been interrupted by treatment of VP6 with anti-VP6 antiserum suggesting VP6 contains a specific site for binding to the core particle (Sandino et al., 1988). Particles analogous to single-shelled particles have been isolated from infected cells and shown to be capable of synthesizing mRNA (Helmer-Jones & Patton, 1986). Comparison of the deduced amino acid sequences of bovine rotavirus VP1 and SA11 rotavirus VP3 with protein sequences of various viral polymerases has revealed conservation within these proteins (Cohen et al., 1989; Liu & Estes, 1989). Temperature-sensitive mutants that map to genes for VP1 and VP3 are defective in ssRNA and dsRNA synthesis, whereas temperature-sensitive mutants that map to genes for VP2 and VP6 are defective in the production of dsRNA (Gombold et al., 1985; Gombold & Ramig, 1987). Studies by Valenzuela et al. (1991) and Pizarro et al. (1991) indicate that VP1 and VP3 are the rotavirus RNA transcriptase and guanylyltransferase, respectively.

Our results, using three MAbS, indicate one or more sites located on VP6 are involved with the transcription process. Each affinity-purified MAb inhibited in vitro rotavirus transcription by greater than 90% when compared to transcription levels produced by virus alone. Adsorption studies to remove antibody substantiated the hypothesis that these rotavirus-specific MAbS blocked or inhibited rotavirus transcription.

The biphasic dose-response curve exhibited by two of the MAbS was unexpected. Perhaps at high antibody concentrations, aggregation of these molecules occurred, limiting interaction with the epitopes involved in transcription. Removal of antibody aggregates by centrifugation may restore inhibition of transcription to these preparations. It has been demonstrated that soluble antigens are precipitated only by optimum concentrations of antibody (Unanue & Baruj, 1984). It may also be possible that the lack of inhibition of transcription was caused by a greater than optimum concentration of antibody. This could be explained by the possibility that certain divalent antibodies may link separate VP6 molecules together. Such bridges could lock the viral particle into a conformation that does not permit RNA synthesis. Thus, at high concentrations of antibody, there could be competition by excess antibody for epitopes on VP6, resulting in little or no bridge formation. With no bridge formation by certain antibodies at high concentrations, RNA synthesis would be free to proceed.

The ability of the antibodies to bind to single-shelled
virus and inhibit transcription indicates that the sites on VP6 are exposed and antigenic. It is possible that these sites may be remote from the active site of the transcriptase. In fact, in a complex structure such as the rotavirus particle, the protein that binds the antibodies may not be the protein possessing the transcriptase activity. Binding of antibody might produce a conformational change that is transmitted to the enzymatic site, interfering with its activity. Conformational changes have been reported to affect the transcriptional activity of reovirus cores (Powell et al., 1984). It has also been suggested that the aqueous channels of single-shelled particles serve as routes for importing the metabolites required for RNA synthesis and exporting the newly synthesized RNA transcripts (Prasad et al., 1988). The MAbs may bind to the virion in a way that prevents the transport of these molecules, thereby inhibiting transcription.

The epitopes located on SA11 virus and recognized by our MAbs were conserved among four different rotavirus serotypes. This suggests that the site(s) involved with transcription are structurally and functionally similar for these viruses. This conclusion is substantiated by a high degree of conservation (≥ 90%) between the amino acid sequences of VP6 from different rotavirus serotypes (Both et al., 1984; Gorziglia et al., 1988).

Inhibition of transcription of heat-activated double-shelled SA11 rotavirus suggests that treatment of double-shelled virus with heat results in a particle with a transcription complex that is structurally and functionally similar to that found in single-shelled rotavirus. These data corroborate findings by Spencer & Garcia (1984). No major differences were observed in the activity of reovirus cores (Powell et al., 1984; Gorziglia et al., 1988). It has also been suggested that the aqueous channels of single-shelled particles are exposed and antigenic. It is possible that these sites may be remote from the active site of the transcriptase. In fact, in a complex structure such as the rotavirus particle, the protein that binds the antibodies may not be the protein possessing the transcriptase activity. Binding of antibody might produce a conformational change that is transmitted to the enzymatic site, interfering with its activity. Conformational changes have been reported to affect the transcriptional activity of reovirus cores (Powell et al., 1984). It has also been suggested that the aqueous channels of single-shelled particles serve as routes for importing the metabolites required for RNA synthesis and exporting the newly synthesized RNA transcripts (Prasad et al., 1988). The MAbs may bind to the virion in a way that prevents the transport of these molecules, thereby inhibiting transcription.

At this time we are unable to conclude whether most or all antibodies to VP6 will block rotavirus transcription. Our screening process involved the testing of eight rotavirus-specific MAbs for the inhibition of transcription. Three MAbs inhibited transcription and when tested by Western blotting were found to be specific for VP6. The specificity of the other five MAbs was not determined. Two commercial rotavirus MAbs to VP6 (Chemicon 851 and 892) did not inhibit transcription when tested at a single undiluted concentration. These negative transcription results for the Chemicon MAbs may be due to a biphasic dose–response. Because of a possible biphasic dose–response, it is important to test MAbs at several concentrations for functional activity before concluding a negative result.

References


Short communication


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