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Production of a monoclonal antibody against the Snow Mountain agent of gastroenteritis by *in vitro* immunization of murine spleen cells

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ABSTRACT **The Snow Mountain agent (SMA) is a 27- to 32-nm noncultivatable virus that causes acute gastroenteritis in humans. SMA is morphologically similar to but immunologically distinct from the Norwalk agent. SMA has been partially purified from the stool of experimentally infected volunteers and contains a single structural protein of M_r 62,000 as well as one or more non-virion-associated soluble proteins. Further characterization of this important human pathogen and other Norwalk-like viruses has been hindered by the lack of reagents with which to study them. To further characterize SMA, we developed a monoclonal antibody to SMA using *in vitro* immunization—a technique that permitted use of small quantities of antigen for immunization. The monoclonal antibody, SM-4, was specific for SMA and did not react with the Norwalk or Hawaii agents. In addition, SM-4 reacted with purified virion but not with the soluble protein. SM-4 also blocked the ability of labeled postinfection human IgG to bind to purified virion. Finally, both SM-4 and human postinfection sera specifically recognized the M_r 62,000 virion-associated protein. Thus, SM-4 is directed against an epitope present on the SMA structural protein that is not shared by the Norwalk or Hawaii agents and that is not present on the soluble protein. The availability of a monoclonal antibody against SMA should facilitate further purification and characterization of this agent. The techniques utilized in these studies provide a method for the production of additional monoclonal antibodies to this group of viruses and also should be useful for the study of other occult viral agents.**

The Norwalk-like viruses are 27- to 32-nm round viruses that have been associated with outbreaks of acute gastroenteritis with high secondary attack rates. Several immunologically distinct agents have been described including the Norwalk agent (1), the Hawaii agent (2), and the Snow Mountain agent (SMA) (3). Radioimmunoassays and enzyme immunoassays (EIA) have been developed for the Norwalk agent (4–7), the SMA (7, 8), and the Hawaii agent (unpublished data). The reagents used in these assays consist of sera and stool specimens from human volunteers or chimpanzees experimentally challenged with these viruses. The Norwalk agent has been established through the use of these assays as an important cause of gastroenteritis throughout the world, and serologic surveys have shown a high prevalence of Norwalk antibody in both developed and developing countries (9). More limited studies with SMA have associated this agent with outbreaks of gastroenteritis in various areas of the continental United States (10–12) and have shown that >50% of adults who have been tested have serum antibody to this virus (7).

These assays also have allowed a partial purification and characterization of the agents from the stools of experimentally infected volunteers. The Norwalk agent, when purified from diarrheal stool, contains a single structural protein of M_r 59,000 (13). In addition, a M_r 30,000 protein that reacts specifically with human postinfection serum but is not associated with virion has been detected in the stools of individuals infected with the Norwalk agent and has been termed "soluble antigen" (13). Similar results have been obtained with the SMA, which contains a single structural protein of M_r 62,000 when purified from the stools of volunteers and a soluble antigen that has not been further characterized (14). The presence of a single structural protein of the above size is characteristic of caliciviruses among known animal viruses (15), but definitive classification of the Norwalk and Snow Mountain agents awaits characterization of their genomes.

Because virus is shed briefly and in small amounts by ill individuals (16) and no practical animal model or *in vitro* culture system is available (17, 18), more extensive characterization of these agents has not been performed. In addition, the limited amounts of available viral antigen have hindered previous attempts to generate animal hyperimmune sera or monoclonal antibodies to these viruses.

Recently, the technique of *in vitro* immunization has been developed for the production of monoclonal antibodies (19). A major advantage of this technique is that much smaller amounts of antigen are required to generate an immune response. This technique has been used to produce monoclonal antibodies to scarce or highly conserved antigens such as calmodulin (20), osteoclast-activating factor (21), and hypothalamic growth hormone-releasing factor (22). We now report the use of this technique to develop a monoclonal antibody to SMA and the use of this antibody in the further characterization of SMA virion and soluble antigen.

MATERIALS AND METHODS

Viruses and Cells. SMA used these in experiments was derived from experimentally infected volunteers, all of whom were ill and subsequently seroconverted to SMA by radioimmunoassay or EIA (3, 7, 8). Feline calicivirus (Bolin strain; VR652) and fetal cat tongue cells (Fc3Tg; CCL-176) were obtained from the American Type Culture Collection, Rockville, MD. Simian rotavirus SA-11 was a gift of A. Z. Kapikian, Bethesda, MD, and MA-104 cells were obtained from M. Menegus, Rochester, NY. X63-Ag8.653 myeloma cells were a gift of D. Viceps-Madore, Rochester, NY.

***In Vitro* Immunization.** SMA antigen for immunization was obtained from the diarrheal stool of an experimentally infected volunteer and purified as described (14). The titer of a 10% (wt/vol) homogenate of this stool specimen prior to purification was 1:80 in the EIA for SMA. Briefly, 20 g of this stool was homogenized with 20 ml of TNE/Triton buffer (0.05 M Tris/0.15 M NaCl/0.01 M EDTA/0.1% Triton X-100,

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Abbreviations: SMA, Snow Mountain agent; EIA, enzyme immunoassay; RICA, radioimmunocapture assay; HRP, horseradish peroxidase; TMB, tetramethylbenzidine.

pH 7.4) and extracted twice with trichlorotrifluoroethane (Genetron 113). The supernatant fluid was precipitated by addition of saturated ammonium sulfate to a final concentration of 60%, and the precipitated proteins were resuspended, layered on a 10–40% (wt/vol) sucrose velocity gradient, and centrifuged at 120,000 × *g* for 60 min. The fractions containing virus were pooled and layered on a 50% (wt/vol) potassium tartrate/30% (vol/vol) glycerol density gradient. This gradient was centrifuged to equilibrium, and the fractions containing virus, corresponding to a density of 1.27–1.29 g/ml, were pooled. The virus then was pelleted and resuspended in culture medium for immunization.

Spleen cells from unprimed female BALB/c mice age 4–6 weeks (Charles River Breeding Laboratories) were collected aseptically, washed in Hanks' balanced salt solution, and resuspended at 1×10^7 cells per ml in serum-free immunization media [Dulbecco's modified Eagle's medium containing 4.5 g of glucose per liter (DMEM) plus 5% NCTC 109 medium (MA Bioproducts), 1 mM sodium pyruvate, 2 mM L-glutamine, 50 mM 2-mercaptoethanol, and 0.3 mM 8-bromoguanosine (23) (Sigma)]. Resuspended antigen was added, and cells were exposed to antigen under serum-free conditions for 8 hr at 37°C in 7% CO_2/93% air (24). After 8 hr, an equal volume of thymocyte-conditioned medium prepared by the method of Reading (19) containing 40% (vol/vol) fetal calf serum was added, and the culture was continued for a total of 3–5 days. On days 3, 4, and 5, an aliquot of spleen cells was removed and fused with nonsecreting X63-Ag8.653 myeloma cells at a 5:1 spleen/myeloma cell ratio by using 40% polyethylene glycol (M_r 1000; Aldrich) and 5% dimethyl sulfoxide in DMEM. Fused cells were adjusted to 1×10^5 spleen cells per ml in HAT medium (100 μM hypoxanthine/230 μM thymidine/0.4 μM aminopterin in DMEM containing 20% fetal calf serum; Sigma) and plated at 100 μl per well in 96-well plates (Costar, Cambridge, MA). Hybridomas were screened by the screening assay described below, and the positive hybridoma (SM-4) was recloned by limiting dilution and injected intraperitoneally in BALB/c mice previously primed with pristane (Aldrich).

Screening EIA. Hybridoma supernatants were screened for antibody to SMA by using a "double-sandwich" assay. Acute and convalescent sera from a volunteer challenged with SMA were adsorbed to alternate wells of 96-well polystyrene plates and then incubated with a crude stool homogenate containing SMA derived from a second volunteer. The wells were washed, and hybridoma supernatant was added, followed by addition of horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (Kirkegaard and Perry, Gaithersburg, MD). Assays were developed with tetramethylbenzidine (TMB) (Aldrich) in which absorbance was read at 450 nm. Supernatants that produced an absorbance in wells coated with convalescent serum twice that in wells coated with acute serum (or a positive/negative ratio of 2) were considered positive.

Antibody Characterization. Antibody subclass was determined by a commercial EIA assay (Chemicon International, El Segundo, CA) and $NaDodSO_4$/polyacrylamide gel electrophoresis of culture supernatant biosynthetically labeled with [^{35}S]methionine (New England Nuclear). Ascites fluid was purified by chromatography on a Sephacryl S-300 column (Pharmacia). Aliquots of purified antibody were biotinylated by reaction with biotin-*N*-hydroxysuccinimide ester (Pierce) for 4 hr at room temperature (25).

Specificity of SM-4 for SMA. The reaction of SM-4 with various antigens was investigated in one of two formats. Biotinylated SM-4 was used directly as indicator in the double sandwich assay described above, and the reaction was developed with avidin-linked HRP (Vector Laboratories) and TMB. To test the ability to detect SMA, acute and convalescent sera from a volunteer challenged with SMA were used as "capture antibodies" (to bind antigen from stool specimens). For testing the reaction with the Norwalk and Hawaii agents, pre- and postinfection sera from volunteers challenged with Norwalk or Hawaii were used as capture antibodies. In the second type of test, SM-4 was used as the capture antibody. SM-4 or a control IgM monoclonal antibody was adsorbed at 1 μg per well, followed by antigen, biotin-labeled human convalescent antibody (Norwalk or SMA), avidin-HRP, and TMB. The control monoclonal IgM used in this test and subsequently was Liab Ia (Litton Diagnostics, Charleston, SC), a mouse IgM monoclonal antibody to the murine histocompatability *I*-region-associated (Ia) antigen. The IgM was purified by polyethylene glycol precipitation and exclusion chromatography. Antigens used in these tests included crude stool homogenates from volunteers challenged with Norwalk, Hawaii, or SMA; fractions of a sucrose velocity gradient containing SMA virion; and soluble antigen as described below. Samples giving an absorbance twice that of control wells (positive/negative ratio of 2) were considered positive. Crossreactivity with feline calicivirus and SA-11 rotavirus was tested by incubating biotin-labeled SM-4 with methanol-fixed infected and uninfected Fc3Tg and Ma104 cells, respectively.

The specificity of SM-4 for SMA was further investigated by blocking EIA (8). Blocking assays were performed with human convalescent sera as the capture antibody, followed sequentially by antigen, the unlabeled antibody being tested, and finally by biotin-labeled second antibody, either human or monoclonal. Tests were developed with avidin-HRP and TMB. After subtraction of the values obtained from unblocked wells coated with acute serum, the results for each dilution were calculated as the percentage of absorbance obtained from the unblocked positive control wells (≈0.7–1.0). The titer of each antibody was calculated as the greatest dilution blocking 40% of the value of the unblocked control. The unlabeled antibodies tested included human acute and convalescent sera from a volunteer experimentally infected with SMA and monoclonal antibodies SM-4 and LiAb Ia. Two antigen preparations were used in the blocking assays. "Virion" consisted of SMA virions purified from the stool of an experimentally challenged volunteer by Genetron extraction, pelleting, and sucrose gradient centrifugation. Soluble antigen was obtained from the supernatant remaining after pelleting the virus from a similar stool preparation, concentrated by ammonium sulfate precipitation, and dialyzed against phosphate-buffered saline (0.05 M PO_4^{3-}/0.15 M NaCl, pH 7.4) for 16 hr at 4°C.

Radioimmunocapture Assay (RICA). SMA virions from another volunteer were purified as described above for *in vitro* immunization and labeled with ^{125}I (Amersham) at a specific activity of 13.75 mCi/μg (1 Ci = 37 GBq) by the chloramine-T procedure. Purified ^{125}I-labeled SMA virions were then diluted with an equal volume of fetal calf serum and added to triplicate wells of a 96-well polyvinyl chloride plate (Dynatech) coated with serial dilutions of SM-4, control monoclonal IgM (LiAb Ia), or pre- and postinfection human sera from a volunteer challenged with SMA. After incubation for 4 hr at 37°C, unbound antigen was removed by washing six times (washing buffer: 0.5 M NaCl and 0.05% Tween 20 in phosphate-buffered saline, pH 7.4), and bound antigen was disrupted and eluted by incubation with Laemmli sample buffer containing 0.2% $NaDodSO_4$ and 0.01 M dithiothreitol for 5 min. Eluted, labeled antigen was then subjected to discontinuous $NaDodSO_4$/PAGE and autoradiographed with Kodak XAR-5 film.

RESULTS

Monoclonal Antibody Production. A crude stool homogenate from a volunteer infected with SMA was selected as a

source of antigen for immunization. This volunteer received a third human passage inoculum and manifested an illness characterized by fever, myalgia, malaise, vomiting, and diarrhea. The stool sample was passed on day 2 of illness, and virus was subsequently purified as described in *Materials and Methods*. The virion peak corresponding to a density of 1.28–1.29 g/ml in potassium tartrate/glycerol was used for immunization. The amount of viral protein contained in this sample could not be measured directly but could barely be visualized by silver staining of a polyacrylamide gel. From this analysis, we estimated that 10–20 ng of M_r 62,000 antigen was available for immunization. This material was exposed to spleen cells of BALB/c mice under serum-free conditions for 8 hr, and then the cultures were maintained for 2–5 days.

Approximately 1150 hybridomas were derived from three fusions of 1×10^7 immunized spleen cells at each fusion—470 on day 3, 450 on day 4, and 130 on day 5. The majority of these hybridomas were negative for antibody, had high background activity, or lost antibody production with time. One hybridoma, SM-4 from the day 3 fusion, was consistently positive in the screening EIA and was expanded, recloned by limiting dilution, and used for further antibody production. SM-4 produced an IgM with κ light chain as determined by EIA and NaDodSO$_4$/PAGE of [^{35}S]methionine-labeled supernatant of the hybridoma culture. SM-4 was purified from ascites fluid by gel chromatography, and aliquots were labeled with biotin.

Reactivity of SM-4. To determine the spectrum of reactivity of SM-4 with the Norwalk-like viruses, we tested stool samples from human volunteers experimentally challenged with the Norwalk agent, Hawaii agent, or SMA. These samples were tested in parallel in the standard enzyme assay with polyclonal human sera and in an enzyme assay in which SM-4 was substituted for the indicator or capture antibody. These results are shown in Table 1. The majority of stool samples containing SMA in the standard assay were also positive when SM-4 was used as either indicator or capture antibody. None of the SMA-negative samples reacted with SM-4, and none of the stool samples containing Norwalk or Hawaii agent reacted with SM-4. In addition, biotin-labeled SM-4 did not react with cell cultures infected with either feline calicivirus or SA-11 rotavirus.

To further investigate the specificity of SM-4 for SMA, SMA virions were pelleted from a stool homogenate containing SMA and applied to a 10–40% sucrose gradient. The fractions from this gradient were tested by EIA with biotin-labeled SM-4 or polyclonal human IgG as indicator antibody. These results are shown in Fig. 1. Both SM-4 and human IgG reacted with fractions corresponding to virion, but SM-4 did not react with the upper fractions containing soluble antigen that reacted with human antibody. To investigate the reaction of SM-4 with soluble antigen, a concentrated soluble antigen

Table 1. Reactivity of monoclonal antibody SM-4 in EIA with stool samples containing SMA or Norwalk or Hawaii agents

Stool samples from volunteers challenged with agents	Result in standard EIA	EIA with SM-4, no. +/no. tested: SM-4 as indicator antibody	EIA with SM-4, no. +/no. tested: SM-4 as capture antibody
SMA	+	10/16	8/14
SMA	−	0/12	0/12
Norwalk	+	0/18	0/8
Norwalk	−	0/13	0/3
Hawaii	+	0/5	NT

Samples from volunteers experimentally challenged with SMA, Norwalk agent, or Hawaii agent were tested by standard EIA (+, no. +/no. tested > 2) using the homologous human sera and by EIA using SM-4 as either indicator or capture antibody. NT, not tested.

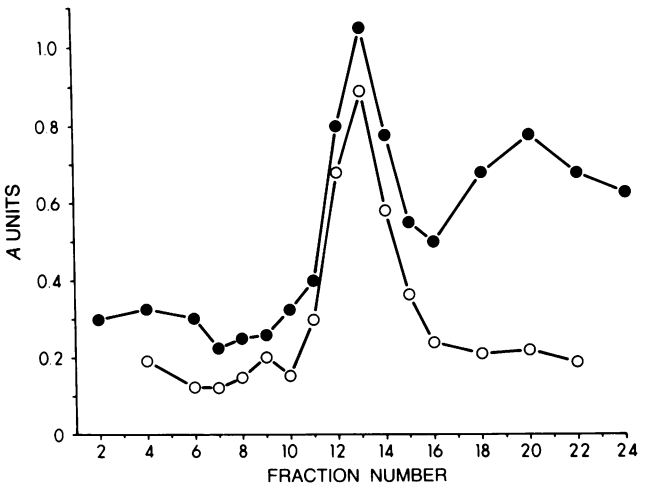

FIG. 1. Diarrheal stool containing SMA was applied to a 10–40% sucrose velocity gradient, and fractions were tested in an EIA with human postinfection serum as the capture antibody and either biotinylated human postinfection IgG (●) or biotinylated SM-4 (○) as the indicator antibody. Both human and monoclonal antibodies show a peak of reactivity with fraction 13, while the human antibody, but not the monoclonal antibody, shows an additional peak at fraction 20.

preparation was made by pelleting the virion from a stool containing SMA, followed by precipitation of the remaining supernatant with ammonium sulfate. The reactivity of human antibody and SM-4 with dilutions of the concentrated soluble antigen is shown in Fig. 2. This preparation of soluble antigen was highly reactive in the standard antigen assay with biotin-labeled human anti-SMA IgG as indicator and had a titer of 1:512. However, when biotin-labeled SM-4 was used as indicator, there was no reaction even at an antigen dilution of 1:4.

To confirm the specificity of SM-4, we tested the ability of unlabeled SM-4 to block the attachment of labeled human postinfection IgG to purified virion prepared by sucrose gradient centrifugation or to concentrated soluble antigen prepared as described above. SM-4 blocked the binding of human IgG to SMA virion and had a titer of 4×10^{-5} (Fig. 3). Despite this high titer, blocking was incomplete, even at a 1:25 dilution of SM-4. The control IgM monoclonal antibody (LiAb Ia) adjusted for similar protein content did not

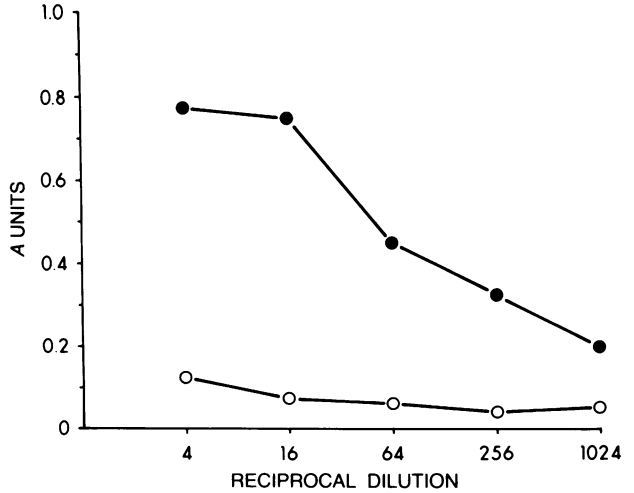

FIG. 2. SMA soluble protein was concentrated by ammonium sulfate precipitation and dialyzed against PBS. Serial 1:4 dilutions of this antigen were tested in an EIA with human postinfection serum as the capture antibody and either biotinylated human postinfection IgG (●) or biotinylated SM-4 (○) as the indicator antibody.

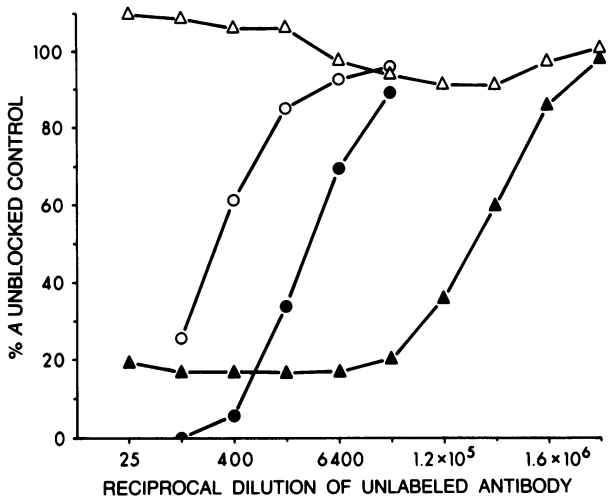

FIG. 3. The ability of unlabeled antibodies to block binding of labeled human IgG to partially purified virion was tested in a blocking EIA. Results at each dilution of unlabeled antibody are expressed as the percentage absorbance compared to the unblocked control. ○, Preinfection human serum; ●, postinfection human serum; △, control IgM monoclonal antibody; ▲, SM-4.

block at any dilution. As an additional control, human postinfection serum blocked at an 8-fold higher titer than did preinfection serum. In contrast, SM-4 did not block the binding of labeled postinfection human IgG to concentrated soluble antigen, although unlabeled human postinfection serum did (Fig. 4). In an additional set of experiments (data not shown), human postinfection serum completely blocked binding of labeled SM-4 to a crude stool homogenate containing SMA.

RICA. To determine the specific SMA protein toward which SM-4 is directed, we tested the ability of SM-4 bound to polyvinyl chloride plates to capture radiolabeled SMA. Bound antigen was disrupted and eluted by incubation with $NaDodSO_4$ sample buffer and analyzed by $NaDodSO_4$/PAGE and autoradiography (Fig. 5). Control wells contained either the control IgM monoclonal antibody LiAb Ia, human prechallenge serum, human postchallenge serum, or no antibody. The partially purified, labeled virion preparation used for this experiment is shown in lane 2 and contained highly labeled bands at M_r values of 51,000, 41,000, and 29,000 as well as less highly labeled bands at M_r 92,000 and M_r 62,000. Both SM-4 (lane 4) and human postinfection serum (lanes 6 and 8) captured a M_r 62,000

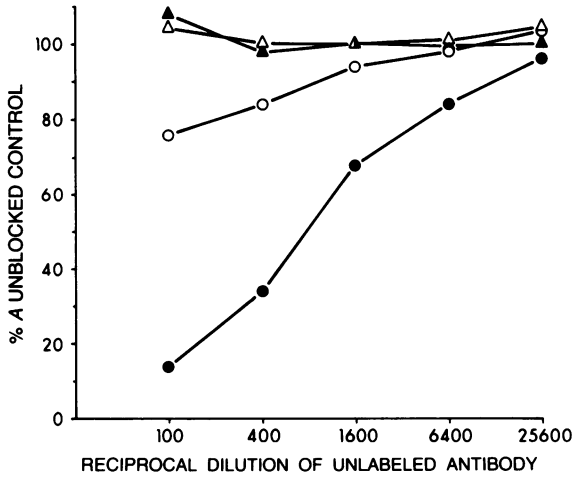

FIG. 4. The ability of unlabeled antibodies to block binding of labeled human IgG to concentrated soluble antigen was tested in the same format as in Fig. 3. ○, Preinfection human serum; ●, postinfection human serum; △, control IgM monoclonal antibody; ▲, SM-4.

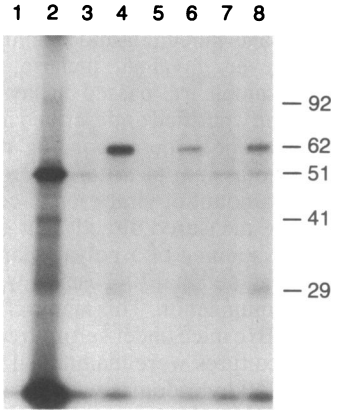

FIG. 5. RICA. Monoclonal antibodies and human sera were diluted in phosphate-buffered saline, absorbed to triplicate wells of a 96-well polyvinyl chloride plate for 6 hr, and then blocked overnight with phosphate-buffered saline containing 1% fetal calf serum and 0.5% gelatin. ^{125}I-labeled SMA was added for 2 hr at 37°C, and unbound virus was washed with phosphate-buffered saline containing Tween 20. Bound virus was disrupted and eluted by incubation with Laemmli sample buffer containing 0.2% $NaDodSO_4$ and 0.1 M dithiothreitol and was analyzed by discontinuous PAGE. Lanes: 1, no antibody; 2, labeled virus; 3, control monoclonal LiAb Ia (5 μg); 4, SM-4 (5 μg); 5, preinfection serum (1:25,000); 6, postinfection serum (1:25,000); 7, preinfection serum (1:3,200); 8, postinfection serum (1:3,200). The position of Coomassie blue-stained molecular weight markers is indicated to the right.

protein, whereas the corresponding control wells did not. A highly labeled M_r 51,000 component was also present but at a much lower activity relative to input and showed no evidence of specific capture. A diffuse band in the range of M_r 29,000–30,000 was also seen in all wells and may have been slightly more dense in the wells containing postinfection serum or SM-4. The significance of this band is unclear because of binding in the control monoclonal antibody wells and in wells containing no antibody (not seen well in this exposure) despite extensive washing. Lack of sufficient viral antigen precluded further attempts to better define this band, and further studies will be required to determine if a minor viral protein or degradation product is present.

DISCUSSION

The anti-SMA monoclonal antibody described in this report, SM-4, is an IgM antibody with κ light chain. SM-4 reacted in a double-sandwich EIA system only with stool samples containing SMA and not with samples containing the Norwalk or Hawaii agents or with cell cultures infected with feline calicivirus or simian rotavirus SA-11. In addition, SM-4 only reacted with those fractions of a sucrose velocity gradient that contained SMA virions and almost completely could block the binding of biotin-labeled human postinfection IgG to partially purified virion. Finally, both SM-4 and human postinfection serum, when bound to wells of a polyvinyl chloride plate, specifically captured the same M_r 62,000 protein in an iodinated, partially purified preparation of SMA virion. Taken together, these data indicate that SM-4 is specific for the single structural SMA protein of M_r 62,000 previously described (14). In addition, the results of blocking assays suggest that SM-4 reacts with an epitope on the SMA virion that is the same or spatially related to the epitope(s) accounting for the majority of the reactivity of the human serum tested. Reaction with an epitope that is densely distributed on the surface of the virion could also account for the results of the blocking assay. The failure of SM-4 to completely block binding of human IgG to the partially purified preparation of SMA virion may be due to residual

contamination of the virion preparation with soluble protein or to the presence of additional epitopes on the SMA virion not recognized by SM-4.

The limited data available suggests that the relative affinity of SM-4 and postinfection IgG for SMA virion were similar. The end-point titer of a stool containing primarily SMA virion by gradient analysis was similar when tested by monoclonal antibody and postinfection serum (data not shown), and the reactivity of purified virions at a single dilution was similar for both antibodies (Fig. 1). In addition, the amount of radiolabeled SMA virion captured by comparable amounts of SM-4 and postinfection IgG were equivalent. Finally, SM-4 at low titer was able to block binding of labeled postinfection IgG to SMA virion. In contrast, SM-4 did not react with soluble antigen at any dilution and did not block the reaction of human convalescent serum with soluble antigen. Thus, soluble antigen does not contain the virion epitope recognized by SM-4. Seen in this light, the failure of SM-4 to react with some samples positive in the serum-based assay may be due in part to the presence of primarily soluble antigen in these samples. Samples containing primarily soluble antigen have been noted before for the Norwalk agent (13), and we were unable to isolate virion from one of the SM-4-negative, human serum-positive samples (data not shown).

The *in vitro* immunization system used in this study has a number of advantages over *in vivo* immunization procedures, including more rapid generation of immune spleen cells and *in vitro* manipulation of cofactors of the immune response (19). This technique is particularly useful when little in the way of purified antigen is available, as was the case with SMA. The amount of SMA antigen used in this immunization was not measured directly but was estimated by visualization on a silver-stained polyacrylamide gel to constitute ≈10–20 ng of the M_r 62,000 protein. However, similar preparations have failed to elicit an antibody response when injected into intact guinea pigs (H.P.M., unpublished data). The development of a system capable of generating an immune response to small quantities of viral antigen along with the recent development of efficient enzyme assays for these agents (5–7) should allow development of monoclonal antibodies to other Norwalk-like viruses. This system may also have application to the study of other occult viral agents that have not been cultivated *in vitro*.

In summary, this report describes the development of an IgM monoclonal antibody, SM-4, specific for the M_r 62,000 SMA structural protein and the use of this reagent to further characterize SMA. The development of monoclonal antibodies to this group of agents will facilitate their purification and characterization, as well as provide reagents for extensive diagnostic and seroepidemiologic studies.

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