Monoclonal Anti-Idiotype Induces Antibodies Against Bovine Q17 Rotavirus

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Received 18 March 1992/Accepted 24 June 1992

This study describes, for the first time, the production and use of an “internal-image” anti-idiotypic monoclonal antibody (MAb) to elicit a rotavirus-specific antibody response. An immunoglobulin G2a MAb, designated RQ31 (MAb1), specific for the outer capsid protein VP4 of bovine Q17 rotavirus and capable of neutralizing viral infection in vitro was used to generate an anti-idiotypic MAb (MAb2). This MAb2, designated RQA2, was selected by enzyme-linked immunosorbent assay (ELISA) using F(ab')2 fragments of RQ31. RQA2 (MAb2) inhibited the binding of RQ31 (MAb1) to the virus but had no effect on the binding of other rotavirus-specific MAbs. The MAb2 also inhibited virus neutralization mediated by MAb1 in a dose-dependent fashion. Naive guinea pigs immunized with the MAb2 produced anti-anti-idiotypic antibodies (Ab3) that reacted with bovine Q17 rotavirus in an ELISA and neutralized rotavirus infection in vitro. The Ab3 response was characterized as MAb1-like because the Ab3 recognizes only the Q17 and neonatal calf diarrhea virus rotavirus strains in ELISA, as did RQ31 (MAb1). The Ab3 response also possessed two other characteristics of RQ31: the abilities to bind the 1.36 (double-capsid) but not the 1.38 (single-capsid) purified rotavirus fraction in ELISA and to immunoprecipitate the VP4 rotavirus protein.

Rotaviruses have been implicated as a cause of diarrhea in children and newborn animals (17). In many countries, rotaviruses are considered the major infectious cause of diarrhea in calves (4, 15, 26, 32, 38). Various approaches to protecting calves against rotavirus-associated diarrhea have been tried. Limited success has been achieved by passive immunization mediated by specific neutralizing antibodies in colostrum and milk (35). This type of immune response appears to occur in vivo, since most cattle are seropositive for rotavirus and have high collostral antibody titers that rapidly decrease to low, nonprotective levels in the milk (1). Intramuscular vaccination with rotavirus and adjuvant has been used to enhance antibody titers in serum, colostrum, and milk (3, 9, 27, 34–36, 42). In most of these studies, collostral antibodies prevented or reduced the severity of diarrhea. However, in only one study was complete protection observed (34). In this particular case, calves were fed with pooled colostrum from cows vaccinated with a live-rotavirus preparation suspended in incomplete Freund’s adjuvant (IFA). Oral immunization of calves is potentially an important approach to rotavirus vaccination, since it would eliminate the need for continuous feeding with colostrum or milk containing high concentrations of specific antibody. Unfortunately, good protection by using this method has not been reported (2, 8, 15).

Neutralizing-antibody production and consequent protection against viral infection are mediated by epitopes on the outer capsid proteins VP4 and VP7 (17). In recent years, new vaccination alternatives for rotavirus, such as the use of synthetic peptides corresponding to conserved regions on VP7 and VP4, have been proposed (22). Similarly, Brüssow et al. (6) reported the enhancement of rotavirus-specific antibodies in the milk of cows vaccinated with a subunit vaccine composed of an empty rotavirus capsid and VP7. Brüssow et al. (7) also proposed the use of the monkey rotavirus SA11 to immunize cows because of the heterotopic response to monotype vaccines (7, 37).

Idiotypic vaccines are another promising alternative to conventional vaccines. Anti-idiotypic antibodies (Ab2) have been classified into four groups (alpha, beta, gamma, and delta) according to their specificities for different regions of the target antibody (Ab1) (21). Only types beta and gamma bind to the antigen-binding site (paratope) of the Ab1. The beta type mimics an antigenic determinant and binds to the Ab1 in a fashion similar to its binding to the epitope of the original antigen. The gamma type binds to a paratope-associated idiotope but bears no structural resemblance to the antigen (21). Ab2, which mimic the antigens, have been tested as immunogens for several viruses, and several of these “internal-image” Ab2 (beta type) have elicited virus-specific humoral and/or cell-mediated immune responses which confer protective immunity against viral infections (5, 16, 18–21, 24, 25, 29, 30, 33, 39, 41). Jackson et al. (23) have demonstrated that it is possible to expand the use of anti-idiotype vaccines for diseases in which the primary target is a mucosal tissue, such as the lung or gut, by using oral immunization.

In this paper, we describe an Ab2 monoclonal antibody (MAb2) which mimics a neutralizing conserved epitope on the VP4 protein of bovine rotavirus type G6. This MAb2 elicited a rotavirus-specific anti-anti-idiotype (Ab3) response in heterogenic species. This response was characterized as MAb1-like, since it was identical to that of the immunizing MAb RQ31.
MATERIALS AND METHODS

Cells and viruses. The bovine rotavirus Q17 strain was isolated and characterized at the Faculty of Veterinary Medicine, University of Montreal (14). The neonatal calf diarrhea virus (NCDV; G6 and PB1 types) and OSU (porcine G5 type) strains were obtained from Linda Saif, Ohio State University, Columbus. The 678 (bovine G8 and PB2 types) strain was obtained from D. Snodgrass, Morelun Research Institute, Edinburgh, Scotland. The viruses were propagated in MA-104 cells in the presence of crystallized trypsin (Sigma Chemical, St. Louis, Mo.) as previously described (11).

Production of MAb2. MAb RO31 is an immunoglobulin G2a (IgG2a) which recognizes a neutralizing epitope on VP4 of the Q17 strain of rotavirus (12). This MAb was used as the immunogen for the production of MAb2. The nonneutralizing rotavirus-specific MAb RO14 (IgG2a anti-VP4) and RO34 (IgG2a anti-VP6) or the bovine herpesvirus 4-specific MAb H17 (IgG1) and the bovine respiratory syncytial virus-specific MAb 90A (IgG2a) produced at the Faculty of Veterinary Medicine, University of Montreal, were used as control MAb in this study (12). Immunoglobulins were purified from RO31 ascitic fluid by using protein A-Sepharose chromatography (Immunopure IgG purification kit; Pierce Chemical Co., Rockford, Ill.) and coupled to keyhole limpet hemocyanin (KLH) with glutaraldehyde, using a protein weight ratio of 1:1 and a final concentration of 0.05% glutaraldehyde (vol/vol) (25% aqueous solution; Sigma). BALB/c mice were immunized with the KLH-RO31 conjugate mixed with the adjuvant Quil A (Cedarlane, Hornby, Canada). Each mouse was injected subcutaneously with 0.2 ml of the mixture, which is equivalent to 50 μg of MAb1 and 25 μg of Quil A. Two more inoculations of the same antigen preparation were administered at 2-week intervals. Five, four, and 3 days before fusion, intraperitoneal injections containing 50 μg of MAb1 coupled to KLH were given. Mouse myeloma cells (NS-1) were fused with spleen cells from immunized BALB/c mice as previously described (11).

ELISA screening for MAb2. Antibody-secreting hybridomas were screened by enzyme-linked immunosorbent assay (ELISA). Microtiter plates (Nunc) were coated with 0.5 μg of purified RO31 F(ab')2 fragments in phosphate-buffered saline (PBS; pH 7.2) per well for 1 h at 37°C. F(ab')2 fragments of a nonrelated MAb IgG2a were used as antigen control. Nonspecific binding sites were blocked by incubating the plates for 30 min at 37°C in the presence of 3% gelatin (Bio-Rad Laboratories, Richmond, Calif.) in PBS. After two washes with PBS containing 0.05% (vol/vol) Tween 20, 100-μl samples of culture supernatants were added, and the plates were incubated for 1 h at room temperature. The binding of MAb2 was detected with an Fc fragment-specific peroxidase-conjugated goat anti-mouse IgG (Jackson Immunoresearch, West Grove, Pa.). The substrate used was 0.05% H2O2, 0.01% 3,3',5,5'-tetramethylbenzidine (Sigma) in 1 M sodium acetate buffer (pH 6).

MAb F(ab')2 fragments were prepared according to the method of Parham (31). Briefly, 4 mg of MAb1 were digested with 0.4 mg of pepsin immobilized on Sepharose beads (Pierce Chemical) in 20 mM acetate buffer (pH 4.1) for 2 h at 37°C. After the digestion was stopped with 1 M Tris-HCl (pH 7), the Sepharose beads were removed by centrifugation and the antibody was dialyzed against PBS (pH 7.2) for 16 h. Undigested antibodies were removed by affinity chromatography on protein A-Sepharose (Pierce Chemical). Fragment purity was assessed by sodium dodecyl sulfate-polyacryl-
oma cells was detected by an anti-species IgG-fluorescein conjugate (ICN). Hybridoma cells producing IgG1 MAb H17 were used as controls.

**Induction of Ab3.** To elicit an Ab3 response in mice, 8-week-old BALB/c mice were immunized with MAb2 coupled to KLH and mixed with the adjuvant Quil A (Cedarslane). Each mouse was injected subcutaneously with 0.2 ml of the mixture containing 50 or 5 μg of MAb2 and 25 μg of Quil A. Four more immunizations of the same antigen preparation were administered at 2-week intervals. Two control mice in the same cage were inoculated in the same way but with only KLH and adjuvant.

Ten guinea pigs were immunized subcutaneously with either 100 or 10 μg of purified MAb2 mixed with adjuvant Quil A or Freund’s adjuvant. Three more immunizations (subcutaneous) were carried out with the same mixture, and two further immunizations were done with 1 ml containing 10^6 MAb2 hybridoma cells with the same adjuvants. Four control guinea pigs, which shared the cage with the others, were inoculated in the same way with the respective adjuvants containing IgG1 MAb H17 hybridoma cells.

**Characterization of Ab3.** The binding patterns of Ab3 against different rotavirus strains were tested by ELISA. Microtitration plates were coated with 0.5 μg of purified rotavirus Q17, NCDV, OSU, or 678 per well. In addition, the 1.36 (double-capsid) or the 1.38 (single-capsid) (17) purified rotavirus Q17 fractions were used to coat microtitration plates. A volume of 100 μl of mouse or guinea pig polyclonal antiserum was added to each well. Ab3 binding to rotavirus was detected by an anti-species (mouse or guinea pig) IgG-peroxidase conjugate (ICN).

The rotavirus-neutralizing activity of the Ab3 was tested by an NF neutralization test as described above.

The abilities of Ab3 sera to inhibit MAb1-MAb2 binding were examined by competitive inhibition ELISA. Purified MAb2 was used to coat microtitration plates at a concentration of 1 μg per well. A volume of 50 μl of peroxidase-labeled MAb1 (dilution, 1/5,000) was mixed with 50 μl of various dilutions of mouse or guinea pig polyclonal Ab3 antisera. The mixture was added to the plate immediately. The percent inhibition of MAb1 binding to MAb2 was calculated as described above.

Ab3 reactivity with rotavirus proteins was detected by immunoprecipitation and SDS-PAGE as previously described (13).

**RESULTS**

MAb RQ31 was selected to generate syngeneic MAb2 because of its neutralizing activity against bovine rotaviruses. The presence of Ab2 in sera from RQ31-immunized mice and hybridoma culture supernatants was demonstrated by ELISA with plates coated with RQ31 F(αb’), fragments. Two clones, 1513 hybridomas were screened for the production of MAb2. Five hybridoma cell lines secreted MAb2 specific for the immunizing antibody. Two stable MAb2, RQA2 and RQA23, were obtained. The isotype of both MAb2 was IgG1. The binding of the biotinylated MAb1 (RQ31) to either of the two MAb2 was competitively inhibited by the other one, indicating that the two MAb2 recognized closely located idiotopes. Further characterizations were performed with only RQA2 MAb2. The RQA2 MAb2 was specific for MAb1 (RQ31), since it did not react with other bovine rotavirus-specific MAb2 (Fig. 1 in F(ab’), ELISA, and only RQ31 was able to inhibit the MAb2-MAb1 reaction in a dose-dependent fashion (Fig. 2). In addition, the MAb2 purified immunoglobulin inhibited MAb1 binding to rotavirus antigens at concentrations lower than 4.8 ng, but it inhibited only 30% of the RQ104 binding at a concentration of 3,000 ng and 14% of the RQ34 binding at a concentration of 15,000 ng. At these concentrations, the MAb1 binding was inhibited by more than 95% (Fig. 3). When a control MAb IgG1 or IgG2a was used as a competitor, inhibition of more than 10% was never observed. RQA2 binds to MAb1 close to the antigen-binding site of the MAb1, since RQA2 was able to inhibit the virus infection-neutralizing activity of the MAb1, as shown in Fig. 4. This inhibition was not the result of nonspecific MAb2 reactions, since the IgG1 H17 control MAb had no effect.

Rabbit and bovine antisera raised against Q17 purified rotavirus did not bind to the MAb2 in ELISA or IF tests.

An investigation of the Ab3 response to RQA2 was carried out in order to determine whether the MAb2 was either a gamma or a beta Ab2. Mice and guinea pigs were immunized...
with RQA2 to elicit an Ab3 immune response. Tables 1 and 2 show that both species produced rotavirus-specific antibodies in a dose-dependent fashion in response to MAb2 immunization. In addition, Quil A and Freund’s adjuvant were equally effective in the production of this Ab3 response. However, the FF neutralization test showed that only guinea pig Ab3 could neutralize rotavirus infectivity. Preimmune sera and sera from mice immunized with KLH-adjuvant or from guinea pigs immunized with the control IgG1 H17 MAb did not bind to or neutralize rotavirus. As expected, the mouse or guinea pig Ab3 were also able to inhibit the MAb1-MAb2 reaction, as demonstrated by a competitive ELISA (Fig. 5). These results suggest that RQA2 is a beta-type Ab2.

In order to further characterize the Ab3 as Ab1-like, their binding patterns against different rotavirus strains and different preparations of purified rotavirus were investigated. The guinea pig Ab3 bound to only Q17 and NCDV rotavirus strains in ELISA, as did RQ31 (Table 3). Like RQ31, guinea pig Ab3 recognized the purified viral band (1.36; double capsid, which contains VP4) but not the 1.38 band (single capsid, which did not contain VP4) (Table 3). Immunoprecipitation of the 35S-labeled rotavirus proteins by the Ab3 showed that VP4 was the only immunoprecipitated protein (Fig. 6). These results indicate that the Ab3 response is very similar to the MAb1 response.

**DISCUSSION**

RQ31 (MAb1) binds to the neutralizing epitope on VP4 of bovine rotavirus and is conformation specific. This antibody was used to produce MAb2, which specifically inhibited the binding of MAb1 to the virus in ELISA and neutralization tests. These results show that the MAb2 binds to MAb1 close to the antigen-binding site of MAb1. This suggests that MAb2 is either a gamma- or beta-type Ab2 (21). However, the rotavirus-specific polyclonal rabbit and bovine antisera

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**TABLE 1.** Rotavirus-specific ELISA binding of Ab3 sera from mice and guinea pigs

<table>
<thead>
<tr>
<th>Animals* and antigen (amt)</th>
<th>Adjuvant</th>
<th>ELISA titer at postimmunization day**</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
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<tr>
<td>Mice</td>
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<tr>
<td>RQA2 (5 µg)</td>
<td>Quil A</td>
<td>≤10</td>
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<tr>
<td>RQA2 (50 µg)</td>
<td>Quil A</td>
<td>≤10</td>
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<tr>
<td>PBS</td>
<td>Quil A</td>
<td>≤10</td>
</tr>
<tr>
<td>Guinea pigs</td>
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<td></td>
</tr>
<tr>
<td>RQA2 (10 µg)</td>
<td>Quil A</td>
<td>≤10</td>
</tr>
<tr>
<td>RQA2 (100 µg)</td>
<td>Quil A</td>
<td>≤10</td>
</tr>
<tr>
<td>H17</td>
<td>Quil A</td>
<td>≤10</td>
</tr>
<tr>
<td>RQA2 (100 µg)</td>
<td>CFA or IFA</td>
<td>≤10</td>
</tr>
<tr>
<td>H17</td>
<td>CFA or IFA</td>
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* In each case, two animals were tested.
** Titers were determined as the reciprocals of the highest dilution giving an ELISA reading of ≥0.1. ND, not determined; †, booster immunization.

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**TABLE 2.** Rotavirus-neutralizing activity of Ab3 sera from mice and guinea pigs

<table>
<thead>
<tr>
<th>Animals* and antigen (amt)</th>
<th>Adjuvant</th>
<th>FF titer at postimmunization day**</th>
</tr>
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<tr>
<td></td>
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<td>Mice</td>
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<tr>
<td>RQA2 (5 µg)</td>
<td>Quil A</td>
<td>≤10</td>
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<tr>
<td>RQA2 (50 µg)</td>
<td>Quil A</td>
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<tr>
<td>PBS</td>
<td>Quil A</td>
<td>≤10</td>
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<tr>
<td>Guinea pigs</td>
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<tr>
<td>RQA2 (10 µg)</td>
<td>Quil A</td>
<td>≤50</td>
</tr>
<tr>
<td>RQA2 (100 µg)</td>
<td>Quil A</td>
<td>≤50</td>
</tr>
<tr>
<td>H17</td>
<td>Quil A</td>
<td>≤50</td>
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<tr>
<td>RQA2 (100 µg)</td>
<td>CFA or IFA</td>
<td>≤50</td>
</tr>
<tr>
<td>H17</td>
<td>CFA or IFA</td>
<td>≤50</td>
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</table>

* In each case, two animals were tested.
** Titers were determined as the reciprocals of the highest dilution neutralizing 100 fluorescent cell-forming units of bovine Q17 rotavirus. ND, not determined; †, booster immunization.

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and whereas rotavirus
Viral MAb2
The sensitivity could be due
the on was used (or present
in the presence of serum from a mouse immunized with 50 μg of RQA2 (MAb2) and Quil A at day 100 (∅), day 75 (∆), or day 0 (×) after immunization.

FIG. 5. Inhibition of binding of Ab1 to Ab2 by Ab3. Plates coated with MAb2 purified immunoglobulins were incubated with peroxidase-labeled RQ31 (MAb1) in the presence of serum from a guinea pig immunized with 100 μg of RQA2 (MAb2) and Quil A at day 100 (∅), day 75 (∆), or day 0 (×) after immunization or in the presence of serum from a mouse immunized with 50 μg of RQA2 (MAb2) and Quil A at day 100 (∅), day 75 (∆), or day 0 (×) after immunization.

did not react with the RQA2 MAb2 by ELISA or IF test. These results imply that the RQA2 is a gamma-type MAb2 or that the MAb1-specific VP4 neutralizing epitope was not present (or not immunogenic) in the rabbit and bovine immunization protocols. This could have occurred because the antigen preparation used in the immunization of these species was different from that used in the immunization of the mice for MAb1 production. In the first case, the antigen used was rotavirus purified on a cesium chloride gradient, whereas rotavirus used for immunization of mice was partially purified on a sucrose cushion. The expression of epitopes on VP4 could be very complicated because of the protease sensitivity of the epitopes and their disposition as complex spikes in the outer capsid. It has been previously demonstrated that the interaction between VP4 and VP7 affects the expression of a neutralizing epitope on VP4 (10). The MAb2 was able to inhibit 30% of the binding to rotavirus of RQ104, a nonneutralizing anti-VP4 IgG2a MAb. This inhibition could be due to some degree of cross-reactivity between the RQ31 and RQ104 epitopes. The epitope mapping of VP4 will allow us to clarify this possibility.

The MAb2 was used to inoculate mice and guinea pigs in order to study the Ab3 response and to characterize the Ab2 as either gamma or beta type. RQA2 was able to elicit a rotavirus-specific Ab3 response in both animals, as demonstrated by ELISA. Only the Ab3 serum from guinea pig neutralized virus infectivity. Since both mice and guinea pigs developed an antirotavirus humoral response, as detected by ELISA, we expected neutralization activity in both samples. The inability of mouse Ab3 to neutralize rotavirus is difficult to explain. The regimen of immunization used to elicit a syngeneic immune response was probably inadequate, since the specific rotavirus antibody levels detected by ELISA remained very low during the course of the experiment. This might explain why we failed to detect any neutralizing activity in the Ab3 mouse sera. However, we were able to detect an Ab3 neutralizing rotavirus-specific response in guinea pig, a heterogenic species. This suggests that the MAb2 described in the present study is a beta-type Ab2 (21, 25). This conclusion is further supported by the fact that guinea pig Ab3 were characterized as MAb1-like by the ELISA reactivity patterns against different rotavirus strains and different preparations of rotavirus as well as by an immunoprecipitation test. Reactivity against various rotavirus proteins would be expected in the case of a nonspecific Ab3 response or if the MAb2 was a regulatory Ab2. Since no reactivity against rotavirus proteins other than VP4 was observed in the sensitive immunoprecipitation assay, these results lead to the conclusion that the beta-type MAb2 described in this study represents a neutralizing epitope on VP4.

The induction of rotavirus-neutralizing antibodies by internal-image-type RQA2, which can be produced in unlimited amounts, makes this MAB2 interesting as a potential immunogen against rotavirus. This antibody could be used for vaccination of cows to develop a transferable passive immunity or for oral vaccination of calves to develop an active local immunity (23, 40). The utilization of Ab2 vaccine for inducing protective mucosal immunity has been demonstrated in a Streptococcus mutans system (23).

### TABLE 3. Comparison of ELISA reactivities of MAb1 and guinea pig Ab3 sera

<table>
<thead>
<tr>
<th>Viral antigena</th>
<th><strong>Reactivityb (mean ± SD)</strong></th>
<th><strong>MAb1c</strong></th>
<th><strong>Ab3d</strong></th>
</tr>
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<tbody>
<tr>
<td>1.36e</td>
<td>0.367 ± 0.031</td>
<td>0.296 ± 0.039</td>
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</tr>
<tr>
<td>1.38f</td>
<td>0.043 ± 0.005</td>
<td>0.095 ± 0.023</td>
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<tr>
<td>NCDV</td>
<td>0.447 ± 0.025</td>
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<tr>
<td>OSU</td>
<td>0.088 ± 0.023</td>
<td>0.097 ± 0.013</td>
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<tr>
<td>678</td>
<td>0.057 ± 0.005</td>
<td>0.084 ± 0.027</td>
<td></td>
</tr>
</tbody>
</table>

a Used to coat the microtiter plate.

b Reactivity was recorded as optical density in the ELISA. Means are from three experiments.

c RQ31 culture supernatant.

d Serum (1:100 dilution) from guinea pig immunized with 100 μg of RQA2 and Quil A at day 75.

e Flotation density of rotavirus Q17 purified fraction (double capsid).

f Flotation density of rotavirus Q17 purified fraction (single capsid).
ACKNOWLEDGMENTS

We thank J. Hamel for helpful discussions and comments on the manuscript. The technical assistance of Marian Sajna and Nancy Chrétien is gratefully acknowledged.

REFERENCES