

Rapid Detection of Human Group C Rotaviruses by Reverse Passive Hemagglutination and Latex Agglutination Tests Using Monoclonal Antibodies

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Reverse passive hemagglutination (RPHA) tests and a latex agglutination test with monoclonal antibodies (MAbs) were developed for the rapid detection of noncultivable human group C rotaviruses. For RPHA tests, two MAbs, MAb 5A12 recognizing the outer capsid and MAb 13A3 recognizing the inner capsid, were separately used for the coating of sheep erythrocytes (SRBCs). Forty-six fecal samples were examined to confirm the practicality of the tests. As a result, there was concordance between the RPHA test with SRBCs coated with MAb 5A12 and polyacrylamide gel electrophoresis of viral RNA (RNA-PAGE) in 44 (95.6%) of 46 samples, while the diagnoses by the RPHA test with SRBCs coated with MAb 13A3 were in complete agreement with those by RNA-PAGE. Furthermore, a latex agglutination test with MAb 13A3 was also developed, and this test was fast enough and sensitive enough to successfully detect the viruses from most fecal samples within 2 min. The present procedures would be useful for the diagnosis of human group C rotavirus infections in clinical laboratories which are not well equipped.

Rotaviruses are the most common causative agent of gastroenteritis in infants and young children in many countries (11). Until recently, it was believed that there was only one rotavirus group and that all rotaviruses shared a group-specific antigen. However, a number of viruses which are morphologically similar to rotaviruses but do not share the group-specific antigen and usual genome profile generally associated with ordinary rotaviruses have been identified (16, 17). These distinct rotaviruses, atypical rotaviruses, are now classified into six groups (groups B, C, D, E, F, and G), whereas the ordinary rotaviruses are termed group A rotaviruses (20).

Group C rotaviruses were first identified as a cause of gastroenteritis in pigs (1, 21). Thereafter, rotaviruses with electropherotypes and antigenic properties similar to the porcine virus were identified in humans (3). Recently, a large-scale outbreak of diarrhea caused by group C rotavirus in schoolchildren in Japan has been reported (13), and others have also observed additional group C rotavirus infections (2, 4, 15, 18, 27). On the other hand, preliminary serological surveys have shown that the percentage of sera from adults and children positive for group C rotavirus antibodies was from 11 to 42% (7, 14, 20). These results suggest that group C rotaviruses may be emerging enteric pathogens in humans.

Detection of human group C rotavirus (CHRV) has usually been done by immune electron microscopy with reference antisera or polyacrylamide gel electrophoresis of viral RNA (RNA-PAGE), because CHRV is noncultivable. Because these methods are complicated and time-consuming, simple and rapid diagnostic procedures may be valuable for the timely construction of appropriate infection control measures in hospitals and for the gathering of epidemiological data to establish the importance of CHRV in human diar-

rheal diseases. We have undertaken a series of studies to simplify the detection of CHRV and to develop rapid diagnostic methods. We previously developed an enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies (MAbs) raised against CHRV (8). In the present study, we adapted reverse passive hemagglutination (RPHA) and latex agglutination (Lx-Ag) tests by using anti-CHRV MAbs to detect CHRV.

MATERIALS AND METHODS

Viruses. The sources of five CHRV isolates antigenically identified by reference sera have been described previously (8). Forty-six fecal samples obtained from patients with acute diarrhea, as well as clinical isolates, had been examined for the presence of rotaviruses by RNA-PAGE (8). Ten percent suspensions of the fecal samples in phosphate-buffered saline (PBS; pH 7.2) were homogenized with a vortex mixer and were centrifuged at $2,000 \times g$ for 10 min. The supernatants, which were referred to as fecal extracts, were subjected to the assays. The Wa strain of human group A rotavirus (AHRV) was propagated in MA104 cells, and the culture supernatant was used.

MAbs. Two selected MAbs were used in the present study. MAb 5A12, whose subclass was immunoglobulin G2b, recognizing the outer capsid of CHRV was described previously (8). MAb 13A3, whose subclass was immunoglobulin G1, was newly established in this study. The methods used for production and selection of hybridomas have been previously described (8). Briefly, splenocytes of BALB/c mice immunized with purified CHRV were fused with murine myeloma XAg63 cells with polyethylene glycol 4000. Hybridomas were screened for the production of anti-CHRV antibodies by an ELISA with the purified virion. Positive cultures were subcloned twice by the limiting dilu-

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tion method. MAbs were purified from mouse ascitic fluids by affinity chromatography as previously described (8).

Characterization of MAbs. The avidity of MAbs to CHR V was checked by our ELISA system (8) by using homologous MAbs as both capture antibodies and detector antibodies. Briefly, wells of a microtiter plate were coated with 100 μ l of each MAb solution (10 μ g/ml). Purified CHR V solution was added to each well, and the plate was incubated at 37°C for 1 h. After washing, 100 μ l of biotin-conjugated MAb (0.5 μ g/ml) identical to the capture antibody was added, and then horseradish peroxidase-conjugated streptavidin solution was dispensed into the wells. Finally, the substrate solution (3,3',5,5'-tetramethylbenzidine in citrate buffer) was added to each well, and the color was measured with a spectrophotometer at a wavelength of 450 nm.

To determine whether MAbs reacted with the outer or inner capsid, the purified CHR V was treated with various concentrations of EDTA (2, 5, and 10 mM) for 15 min at room temperature. The treated virus was then tested by the ELISA system used for determination of the avidity of the MAbs.

Preparation of MSRBCs. A 2.5% suspension of sheep erythrocytes (SRBCs) fixed with glutaraldehyde was incubated with an equal volume of 0.005% tannic acid solution for 15 min at 37°C. The tanned SRBC suspension was mixed with an equal volume of anti-CHR V MAbs in 0.15 M phosphate buffer (pH 6.4), and the mixture was incubated at 37°C for 15 min. The MAb-coated SRBCs (MSRBCs) were suspended in PBS containing 1% heat-inactivated normal rabbit serum. SRBCs coated with normal mouse immunoglobulin G in the same manner were used as the control.

RPHA tests. To eliminate nonspecific reactions, fecal extracts were preincubated with SRBCs fixed with glutaraldehyde at 37°C for 1 h. After centrifugation, the supernatants were subjected to the RPHA tests by using V-shaped microtiter trays. Serial twofold dilutions of the specimens in 25 μ l of normal rabbit serum-PBS were made in duplicate. In one dilution series, 25 μ l of a 0.7% suspension of MSRBCs was added to each well. In the other series, the same amount of control SRBCs was added. After shaking, the tray was allowed to stand at room temperature. Hemagglutination patterns were observed after 1 h. The reciprocals of the end point dilutions of hemagglutination with MSRBCs or control SRBCs were referred to as RPHA titers. The RPHA index was then defined as follows: RPHA index = RPHA titer with MSRBCs/RPHA titer with control SRBCs. If the RPHA titer was <4, it was regarded as 2. An RPHA index of \geq 4 was judged to be CHR V positive.

Preparation of MAb-coated Lx. Latex particles (Lx) (Takeda Co. Ltd., Osaka, Japan) were coated with MAb 13A3 according to the manufacturer's instructions. Briefly, a 0.5% suspension of Lx in 0.1 M Tris-HCl, pH 8.0 (TB), was incubated with an equal volume of the MAb in TB at 37°C for 1 h with continuous shaking. The MAb-coated Lx (MLx) were washed with TB and resuspended in TB containing 1% normal rabbit serum. The 1% suspension of MLx thus made was kept at 4°C until use. Lx coated with normal mouse immunoglobulin G were used as the control.

An Lx-Ag test. Two aliquots of 10 μ l of fecal extracts were placed on a slide, and equal volumes of MLx or control Lx were added just beside each drop. The drops were carefully mixed with a plastic rod. The slide was then gently tilted by hand during the reaction time. After 2 min, agglutination was macroscopically observed. The specimens which developed agglutination with MLx but not with control Lx were judged to be CHR V positive. When the specimens developed non-

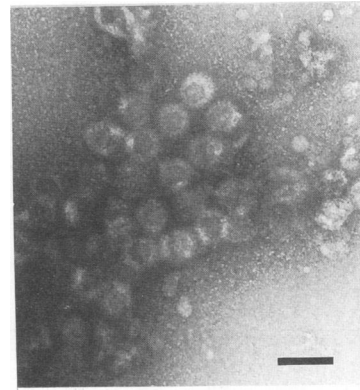


FIG. 1. Immune electron micrograph of EDTA-treated (single-shelled) CHR V incubated with MAb 13A3. Bar, 100 nm.

specific agglutination, they were tested again after treatment with trichlorotrifluoroethane (Daiflon S-3; Daikin Co. Ltd., Osaka, Japan).

Sensitivity of RPHA and Lx-Ag tests. The sensitivity of the RPHA and Lx-Ag tests was determined as described previously (8). The number of virus particles in the purified virus solution of CHR V was counted by electron microscopy, with Lx as a reference, and the serial twofold dilutions of the solution were examined by these methods.

RESULTS

Characterization of MAbs. The avidity of the MAbs to CHR V was checked by the ELISA system (8). The reactivity of MAb 13A3 (optical density, 0.782) was slightly superior to that of MAb 5A12 (optical density, 0.567).

The antigenic site of the MAbs was determined by ELISA reactivity with double- or single-shelled virus particles. The reactivity of MAb 5A12 was decreased after EDTA treatment of the virus, whereas that of MAb 13A3 remained at a constant level or increased. These results suggest that MAbs 5A12 and 13A3 recognize the outer and inner capsids of CHR V, respectively. To verify this conclusion, immune electron microscopy with single-shelled virus was performed. The EDTA-treated CHR V solution (25- μ l volumes) was mixed with an equal volume of each MAb solution (1 μ g/ml). Drops of the reaction mixture were placed on grids stained with uranyl acetate. As a result, MAb 13A3 strongly agglutinated the single-shelled viruses (Fig. 1), whereas MAb 5A12 failed to agglutinate them (data not shown).

Detection of CHR V by RPHA tests. To optimize the conditions for preparation of sensitized erythrocytes, SRBCs coated with either of the MAbs at various concentrations were reacted with serial twofold dilutions of partially purified fecal samples containing CHR V. For both MAbs, the RPHA titer reached a plateau at a concentration of 80 μ g/ml. SRBCs coated with MAb 5A12 (MR5A12) and with MAb 13A3 (MR13A3) at this concentration were used thereafter.

Five clinical isolates which had been identified as CHR V with reference antisera and the Wa strain of AHRV were first tested to check the specificity of these tests (Fig. 2). Four of the five CHR V isolates were positive by the RPHA test with MR5A12, whereas all of them were positive by that with MR13A3. The Wa strain did not react with either MSRBC. The specificity of the tests was also confirmed by a blocking test. The CHR V isolates were incubated with each MAb solution (100 μ g/ml) for 1 h at room temperature, and

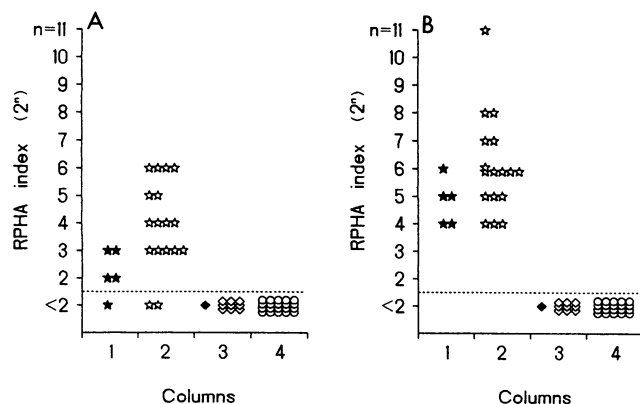


FIG. 2. RPHA indices of the clinical isolates and fecal samples with MR5A12 (A) and MR13A3 (B). Column 1, 5 clinical isolates (*) of CHR V identified with reference antisera; column 2, 17 fecal samples (*) known to contain CHR V; column 3; AHRV samples (◆, Wa strain; ◇, 9 fecal samples containing AHRV); column 4, 20 fecal samples (○) in which no rotavirus was detectable. Isolates and fecal samples with an RPHA index of 4 (2^2) or more were considered CHR V positive.

then the mixture was tested by the RPHA tests. With either MSRBC, the RPHA titers of the samples were drastically reduced by pretreatment with homologous MABs.

To evaluate the practical usefulness of the RPHA tests, fecal samples obtained from patients with acute diarrhea were examined by the tests and the results were compared with those found by RNA-PAGE (Fig. 2). The RPHA titers with MR13A3 were generally higher than those with MR5A12. There was concordance between the RPHA test with MR5A12 and RNA-PAGE in 44 (95.6%) of 46 samples, while the diagnoses by the RPHA test with MR13A3 were in complete agreement with those by RNA-PAGE.

Detection of CHR V by an Lx-Ag test. To further simplify the detection of CHR V, the Lx-Ag test with MAB 13A3 was developed. The optimum condition in which to prepare MLx was studied by the same manner used in the RPHA system. The concentration of MAB showing the most sensitive detection was found to be 1,200 $\mu\text{g/ml}$.

Table 1 shows the detection of CHR V from five clinical isolates and 46 fecal samples by the Lx-Ag test. Specific agglutination was observed within 30 s in 21 of 22 samples known to contain CHR V by either reference sera or RNA-PAGE. Nonspecific agglutinin in a CHR V isolate and an AHRV sample could not be eliminated, even after treatment of these samples with trichlorotrifluoroethane.

Sensitivity of RPHA and Lx-Ag tests. The minimum numbers of CHR V detectable by the RPHA tests with MR5A12 and MR13A3 were 5.5×10^7 and 1.5×10^7 particles per ml,

TABLE 1. Detection of rotaviruses by the Lx-Ag test

| Rotavirus detected by RNA-PAGE (no. of samples) | No. of Lx-Ag test results | | ND ^a |
|---|---------------------------|----------|-----------------|
| | Positive | Negative | |
| CHR V (22) ^b | 21 | 0 | 1 |
| AHRV (9) | 0 | 8 | 1 |
| None (20) | 0 | 20 | 0 |

^a ND, could not be determined (because of nonspecific agglutination).

^b Five clinical isolates and 17 fecal samples.

respectively. In contrast, 3.0×10^8 particles per ml were detectable by the Lx-Ag test.

DISCUSSION

In the present study, we employed RPHA and Lx-Ag tests, which had been widely used in AHRV detection (9, 24, 25), for the specific detection of CHR V. These tests have several obvious advantages over the ELISA that we had established previously, although the ELISA is simple and sensitive compared with electron microscopy or RNA-PAGE (8). The first advantage is detection time. It takes only 2 min to detect CHR V by the Lx-Ag test and 3 h by the RPHA tests, whereas it takes at least 2 days by the ELISA. The second advantage is the simplicity of the test systems. Once SRBCs and Lx coated with MAB are ready, the reactions involve a single step with these methods. Furthermore, no special equipment is required for the Lx-Ag test, and the only equipment required for the RPHA tests is a microtitration set. Consequently, because the present procedures can be used in small clinical laboratories which are not well equipped, they would be valuable for epidemiological studies to define the distribution of CHR V in the world and to establish its importance in human diarrheal disease. Although the sensitivity of these tests is not comparable with that of the ELISA, it is sufficient for routine diagnostic requirements in clinical settings.

When we considered the application of RPHA and Lx-Ag for CHR V detection, the most important issue was the selection of antibodies. The possible sources of group C rotavirus antigens for the preparation of polyclonal antibodies are cultivatable animal group C rotaviruses, such as the Cowden strain (22) and the Shintoku strain (26). In the detection of AHRV by the immunological methods, however, it has been reported that antibodies raised against human rotavirus are superior to ones raised against other animal rotaviruses (6, 23, 28, 29). In fact, the antigenic diversity between human and animal group C rotaviruses has been demonstrated on the basis of their reactivity to antisera in immune electron microscopy analysis (3, 18). Recently, the genetic diversity among group C rotaviruses has also been reported (10, 19). Because no CHR Vs have been successfully propagated in vitro, it is difficult to constantly prepare polyclonal antibodies against CHR Vs. Therefore, we used MABs raised against CHR Vs once they were established; the supply is unlimited and the quality control of diagnostic systems is easy.

The key diagnostic reagent in the present study was MAB 13A3, which recognized the inner capsid of CHR V, because the RPHA test with MR13A3 provided us with more sensitive detection of CHR V and the Lx-Ag test with MAB 13A3 successfully detected CHR V within 2 min. Furthermore, our preliminary data suggest that MAB 13A3 may cross-react with both bovine and porcine group C rotaviruses (data not shown). If testing of additional isolates from animals confirms this cross-reactivity, MAB 13A3 could be useful in monitoring the postulated transmission of group C rotaviruses between humans and animals. Although our RPHA and Lx-Ag tests with MAB 13A3 are promising diagnostic methods, antigenic variation in the epitope on the inner capsid must be taken into consideration. Group A rotaviruses have been classified into subgroups 1 and 2 by the antigenicity of the inner capsid protein (VP6) (11, 12). Cooke et al. (5) have reported that the major inner capsid protein sequence alignments revealed a region of 10 amino acids which were significantly different between human and por-

cine group C rotaviruses. More specimens must be examined before the present procedures can be evaluated conclusively.

In conclusion, our RPHA and Lx-Ag tests were demonstrated to be simple and rapid procedures for diagnosing CHRV infection. The Lx-Ag test is recommended as a screening test, and the RPHA tests are suitable for more precise and quantitative determinations.

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