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Estimation of Rotavirus Immunoglobulin G Antibodies in Human Serum Samples by Enzyme-Linked Immunosorbent Assay: Expression of Results as Units Derived from a Standard Curve

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A method for estimating rotavirus immunoglobulin G (IgG) antibodies by assay of human serum samples at a single serum dilution was studied. Antibody was measured by enzyme-linked immunosorbent assay (ELISA). The optical density of the reaction with a 1:100 dilution of each serum was expressed as ELISA units of antirotavirus IgG by reference to a standard curve. This standard curve was obtained by incorporation in each assay of five dilutions of a serum containing an aribitrary number of units of antirotavirus IgG. Test serum samples found to contain high amounts of antirotavirus IgG were reassayed at a 1:1,000 dilution. There was good correlation between antirotavirus IgG ELISA units in 45 serum samples and endpoint titers of the same samples (Spearman rank correlation coefficient rs, 0.95). Seroconversion during rotavirus infection was defined as an increase in antirotavirus IgG ELISA units per milliliter of greater than 28% (2 × intra-run coefficient of variation of the assay) in consecutive serum samples from the same child. Paired serum samples from nine children with diarrhea not due to rotavirus infection showed no seroconversions. Paired samples from eight children with rotavirus infection showed seroconversions. Estimation of antirotavirus IgG ELISA units in serum is simple, rapid, reproducible, and economical of serum samples. Standardization of results could be achieved by worldwide distribution of a standard serum. Its use would facilitate epidemiological surveys to evaluate potential rotavirus vaccines.

Rotaviruses infect the gastrointestinal tracts of humans and of many animal species. In humans, the clinical and serological consequences of infection are influenced by age. For example, rotavirus infection in newborn babies is often asymptomatic, and persisting serum antibodies are not always detectable after infection (11). Infection in young children is usually associated with acute diarrhea, which can be life-threatening. Serum antibodies appear rapidly and are probably long lasting (11–13). However, possession of serum antibodies does not necessarily confer immunity. Infection with rotavirus is common in older children and adults, and the titer of serum antibody is boosted during symptomatic and asymptomatic reinfection (10, 17). It is possible to detect subclinical or asymptomatic infection in an individual by estimation of the titer of rotavirus antibodies in serum samples taken at regular intervals over several years.

As part of a longitudinal study to determine whether neonatal rotavirus infection conferred immunity against postneonatal infection (1), we decided to moniter sequential changes in the amounts of antirotavirus immunoglobulin G (IgG) in serum samples obtained from children at 3- to 6-month intervals. A total of 106 children were enrolled at birth and were followed for periods ranging from 6 to 36 months. Serum samples were assayed by enzyme-linked immunosorbent assay (ELISA) with simian rotavirus SA11 as antigen (15, 18). The usual method for quantitating immunoglobulin levels by ELISA is to perform an endpoint titration with doubling dilutions of serum.

We decided to seek a simpler method to estimate the amount of antirotavirus IgG in the large number (>1,000) of serum specimens collected during this longitudinal study. The standard curve method is a simple approach to the quantitation of serum antibodies (7) and has been applied successfully to the assay of IgG antibodies against influenza A virus, respiratory syncitial virus, cytomegalovirus, mumps virus, adenovirus, and herpes simplex virus (6, 14, 16). Test serum samples are assayed at a single dilution, and results are converted to units by reference to a standard serum titrated during each assay. We decided to develop a standard curve method to assay antirotavirus IgG by ELISA and to validate the method by comparison with titers estimated by ELISA endpoint titration and complement fixation.

MATERIALS AND METHODS

Patients. Serum samples were collected from 106 children who participated in an epidemiological survey of rotavirus infection. All children were enrolled at birth in one obstetric hospital in Melbourne, Australia. Specimens of cord blood and maternal serum were obtained. Blood was then routinely collected by heel prick or finger prick at the ages of 14 to 21 days and 3, 6, 9, 12, 18, 24, 30, and 36 months. If the child developed acute diarrhea, blood was collected by finger prick at 1 to 6 days and 21 to 28 days after the onset of symptoms. Blood was allowed to clot at room temperature after collection, and serum was separated by centrifugation. Serum samples were stored at −70°C for various lengths of time and were assayed as sets of samples from each child. Feces specimens were collected daily for the first 14 days of life and during episodes of acute diarrhea. Feces were examined for presence of rotaviruses and noncultivable viruses by electron microscopy (2). Portions of feces were also cultured routinely for bacterial enteropathogens.

Serological assays. Antigens. Simian rotavirus (SA11) was grown in an African green monkey kidney cell line (CV-1). Cells were originally grown in Hanks medium containing 10% bovine serum albumin, 1 μg of penicillin per ml, and 0.5 μg of streptomycin per ml. Maintenance medium after

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inoculation with SA11 did not contain bovine serum albumin or antibiotics. Maximum cytopathic effect was evident 2 to 3 days after inoculation. Virus was then extracted by homogenization with an equal volume of trichlorotrifluoroethane (Arklone) in a low-speed blender. The mixture was centrifuged at 10,000 × *g* for 30 min at 4°C to separate the phases. The fluorocarbon phase was reextracted with 2 volumes of phosphate-buffered saline (PBS) and centrifuged again. The supernatant fluids were pooled and centrifuged at 100,000 × *g* for 1 h at 4°C. The pellets containing virus and some cell debris were suspended in 2 volumes of 0.002 M Tris buffer at pH 7.0. This suspended virus was passed through a 0.43-μm Millipore filter and was stored in working volumes at −70°C. Control antigen was prepared in an identical manner using uninfected CV-1 cells and was used at the same dilution as SA11 antigen. The standard concentration of antigens to be used in ELISA was determined by checkerboard titration to assess optimal dilutions.

ELISA conjugate and substrate. Dako anti-human IgG (Dakopatts A/S, code no. P214) raised in rabbit and conjugated with horseradish peroxidase was used as conjugate. The optimum conjugate dilution was determined by checkerboard titration. Before use, conjugate was diluted in PBS-Tween with 1% bovine serum albumin. The substrate used was 80 mg of orthophenylenediamine (British Drug Houses no. 2949) dissolved in 2 ml of methanol and added to 98 ml of citrate phosphate buffer (49.7 ml of 0.05 M citrate and 48.3 ml of 0.1 M Na_2HPO_4). Hydrogen peroxide (0.1 ml of 3% solution) was added to the reaction mixture immediately before use.

Performance of ELISA. The ELISA was performed in Cooke Polyvinyl round-bottomed microtiter plates. The outer rows and columns were not used. Two hundred microliters of SA11 antigen or control antigen diluted in 0.06 M carbonate buffer (0.045 M $NaHCO_3$, 0.018 M Na_2CO_3) was added to each of the inner wells, and the plate was incubated covered at 37°C for 4.5 h and then overnight at 4°C. Plates could be kept for up to 10 days at 4°C with the antigen mixture left in them. When required for an assay, this fluid was aspirated, and the wells were refilled and washed four times with PBST (PBS, pH 7.2, with 1% [wt/vol] Tween).

After the final preparatory washing, 50 μl of each serum dilution to be tested was added to a well. Serum samples were diluted in PBS-Tween containing 1% bovine serum albumin. All test serum samples were assayed at a standard dilution of 1:100, except for maternal and cord serum samples, which were assayed at a standard dilution of 1:200. Dilutions of standard control serum samples included in each assay are listed below. All serum samples were tested in duplicate against the SA11 extract and singly against the control extract. A blank, consisting of the diluent only, was also included on each plate. Control wells containing no serum samples were also included in each assay. After the addition of serum, plates were incubated for 2 h at room temperature and then washed four times with PBST. Fifty microliters of conjugate dilution was then added to each well. The plate was incubated at room temperature for 2 h and then washed with distilled water.

Fifty microliters of enzyme assay solution was then added to each well, and the reaction was allowed to proceed at room temperature in the dark for 15 min. Fifty microliters of 8 N H_2SO_4 was added to each well to stop the reaction. The optical density (OD) of each well was read at 492 nm with a Titertek Multiskan reader blanked on the empty wells of the left-hand column. The OD remained stable over several days, provided the plates were kept in the dark.

Standard sera. Three standard sera were assayed routinely during each day's test. These sera were selected after initial screening of a large number of serum samples from children and adults. The sera were chosen after ELISA endpoint titration showed them to contain either no rotavirus antibodies or antirotavirus IgG in high or moderate amounts.

Serum A was obtained from an adult laboratory worker 21 days after an attack of diarrhea due to rotavirus infection. This serum had an ELISA endpoint titer of 1:128,000. It was arbitrarily decided that this serum contained 20,000 U of rotavirus antibody per ml of serum. Serum A was incorporated in each ELISA assay at doubling dilutions ranging from 1:400 to 1:6,400 (i.e., from 50 to 3.125 U per ml). This range of dilutions corresponded to the linear portion of the curve when OD was plotted against units per milliliter (4).

Serum B was taken from a 2-year-old child 21 days after the onset of acute diarrhea due to rotavirus infection. The serum had an ELISA endpoint titer of 1:2,400 and was incorporated in each assay at a dilution of 1:400.

Serum C was taken from a 12-month-old child 21 days after the onset of acute diarrhea not due to rotavirus infection. This serum had an ELISA endpoint titer of less than 1:50 and was incorporated in each assay at a dilution of 1:100.

CF test. The complement fixation (CF) test was performed by a microtitration method (3). All CF tests were performed by R. C. Pringle, Fairfield Hospital, Melbourne, Australia. Serial twofold dilutions of serum samples beginning with a dilution of 1:4 were reacted with 2 U of antigen, 3 × 50% hemolytic doses of complement, and sensitized sheep erythrocytes (11). Serum samples that gave titers of less than 1:4 were regarded as negative. A fourfold or greater increase in CF antibody titer between two consecutive serum specimens was considered diagnostic of rotavirus infection.

RESULTS

Reproduciblity of the assay. Five different dilutions of the standard positive serum (serum A) were tested 20 times each during one assay. This was repeated on three different days. The coefficients of variation of rotavirus antibody unit values were calculated for each dilution and ranged from 5 to 14%. The intra-run coefficient of variation of the assay was taken as 14%, i.e., as the highest of these values.

To assess inter-run variations inherent in the test, the results from a single dilution of serum were tested in duplicate repeatedly in assays over many months. The inter-run coefficient of variation was 18%.

Determination of OD used to calculate amount of antibody. In all assays, the OD reading of the blank well was subtracted from the OD reading of each test serum sample. This corrected OD value was used to calculate both unit value and endpoint titer. Reactions were taken to be specific for each serum only if the corresponding well containing uninfected control antigen showed an OD reading of <0.07.

A positive-negative cutoff point was used to determine the endpoint of a titration. This cutoff point was estimated after assay of 98 serum samples obtained from 27 children aged 12 months or more. None of the children had been infected with rotavirus as neonates or in the months preceding the date on which each serum specimen was obtained. Serum samples obtained before 12 months of age was not used to estimate the cutoff point since they contained residual amounts of maternal antibody. OD results from assay of these 98 serum samples at a dilution of 1:100 allowed the positive-negative cutoff to be set at the 95th percentile at an OD of 0.1. This

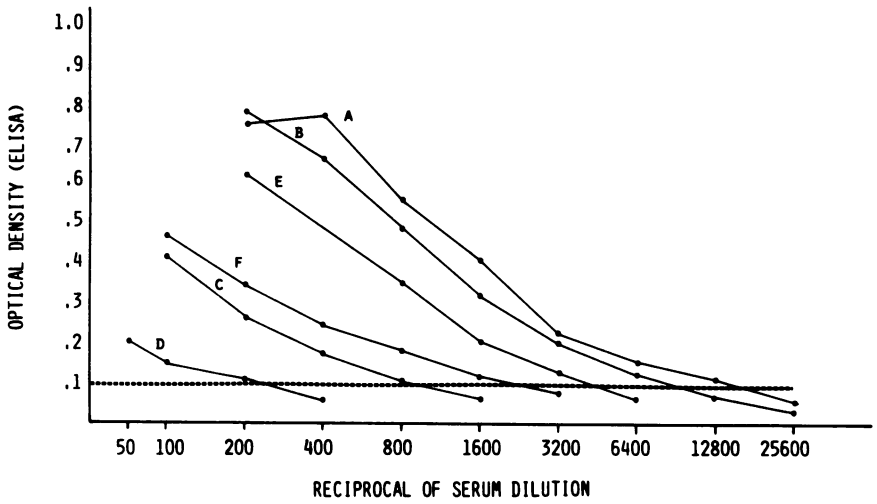

FIG. 1. OD (ELISA) in relation to dilution for consecutive serum samples obtained from one child from birth to 20 months of age. Horizontal dotted line indicates positive-negative cutoff value for OD. (A) At birth: IgG titer, 12,800 (9,900 IgG U/ml); (B) 2 weeks: IgG titer, 6,400 (6,600 IgG U/ml); (C) 6 months: IgG titer, 800 (600 IgG U/ml); (D) 9 months: IgG titer, 200 (<300 IgG U/ml); (E) 15 months: IgG titer, 3,200 (2,800 IgG U/ml); (F) 24 months: IgG titer, 1,600 (800 IgG U/ml).

was used to determine the titer by plotting the OD of each dilution of serum tested against the reciprocal of the dilution (Fig. 1). The lowest dilution giving a OD reading of >0.1 was taken as the endpoint of the specific reaction and was regarded as the endpoint titer. Titration curves for a set of six serum samples obtained from one child from birth to 20 months of age are shown in Fig. 1. Maternally derived rotavirus antibody present at birth (Fig. 1A) declined during the first 9 months of life. Rotavirus infection occurred between 9 and 15 months of age (Fig. 1D and E).

Preparations of standard curves and determination of ELISA units. Serum A was incorporated in duplicate in all assays at doubling dilutions from 1:400 to 1:6,400. Antirotavirus IgG ELISA units contained in these dilutions ranged from 50 to 3.125. During each assay, OD values for each dilution of serum A were plotted against units of antibody present in each dilution. A line of best fit was drawn through these points (Fig. 2). OD values of test serum samples were converted into the equivalent ELISA units by reference to this standard curve. If the initial OD reading of the test serum fell above the highest point on this standard curve, then the serum was diluted further, and the test was repeated. Initially, if the OD reading fell below the lowest point on the curve, the serum was retested at higher concentration (1:10 dilution). It was found that numerous false-positive reactions occurred at this serum concentration, so that this practice was discontinued. A positive-negative cutoff value for units of antirotavirus IgG was estimated after assay of 98 serum samples containing no rotavirus antibodies (described above). The positive-negative cutoff value for antirotavirus IgG ELISA units was set at the 95th percentile, estimated at <300 U/ml of serum.

During each assay, the OD values of control serum B and negative control serum C were converted into ELISA units.

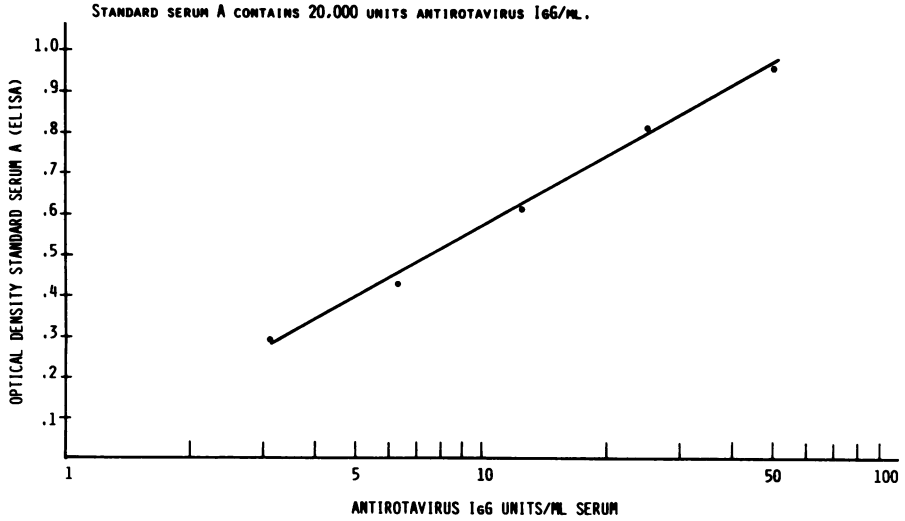

FIG. 2. Standard curve for serum A assayed for antirotavirus IgG (ELISA) with simian rotavirus (SA11) as the antigen. Points are plotted on semilogarithm paper and correspond to doubling serum dilutions (from right to left) from 1:400 to 1:6,400.

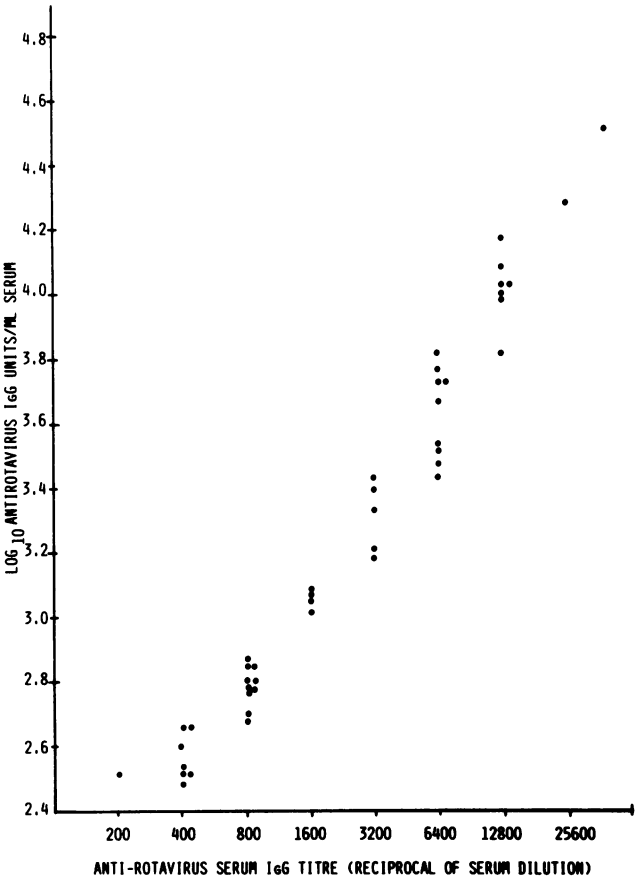

FIG. 3. Antirotavirus IgG ELISA units per milliliter compared with endpoint titers of antirotavirus IgG in 45 serum samples.

If serum B gave a unit value result that fell outside the inter-run coefficient of variation of the assay (18%), then all results of that assay were discarded. If the value for the negative control serum C exceeded 300 U, then all results of the assay were discarded.

Assessment of seroconversion. Seroconversion detected by CF or ELISA endpoint titration was defined as a fourfold or greater increase in titer between two consecutive serum samples from the same individual. Seroconversion detected by the standard curve method was defined as an increase in ELISA unit values per milliliter of more than 28% (2 × intra-run coefficient of variation of the test) in two consecutive serum samples from the same child.

Comparison of results of ELISA IgG units, ELISA IgG titers, and CF titers. Forty-five serum samples containing high, medium, or low amounts of rotavirus antibody were assayed in parallel by standard curve method and by endpoint titration. Log_{10} of ELISA units were plotted against ELISA endpoint titers (Fig. 3). There was good correlation between results obtained by both techniques (Spearmans rank correlation coefficient rs, 0.95; $P < 0.01$). All seroconversions identified in paired serum samples by standard curve assay corresponded to a fourfold or more change in titer by endpoint titration. All seroconversions detected by endpoint titration were also detected by standard curve method.

ELISA IgG units of rotavirus antibody and CF titers were compared for 13 serum samples (Fig. 4). There was good correlation between results of these two assays (rs, 0.95; $P < 0.01$).

The validity of the criteria for assessing seroconversions by the standard curve method was tested in paired serum samples from eight children who excreted rotaviruses (detected by electron microscopy of diarrheal feces) and paired serum samples from nine children with acute diarrhea in whom no excretion of rotavirus was detected (Fig. 5). Diarrhea in the latter nine children was due to infection with adenoviruses (3 children) small viruses (2), *Salmonella* spp. (2), or unknown causes (2). None of the nine control children showed seroconversion. Unit values in consecutive serum samples from these children showed either no changes (7 children) or slight decreases (2). All eight children with rotavirus infection seroconverted. Seven of the acute-phase serum samples had <300 ELISA IgG U/ml, and these values rose to 500 to 2,200 U/ml. One child had an initial value of 500 U/ml that showed a rise to 1,000 U/ml after infection.

DISCUSSION

Within 1 year of the first reports of rotavirus infection in children with acute diarrhea, serological tests were developed to show specific antibody responses after infection (13). Even though human rotaviruses remained resistant to cultivation, serum antibody levels could be measured with cultivable strains of rotaviruses of animal origin. Initially, most tests utilized a bovine rotavirus (Nebraska calf diarrhea virus) that shared some antigens with human rotavirus (8) but was less sensitive than human rotaviruses in detecting antibodies in human serum (12). Eventually, the most widely used serological assay for rotavirus antibodies in human

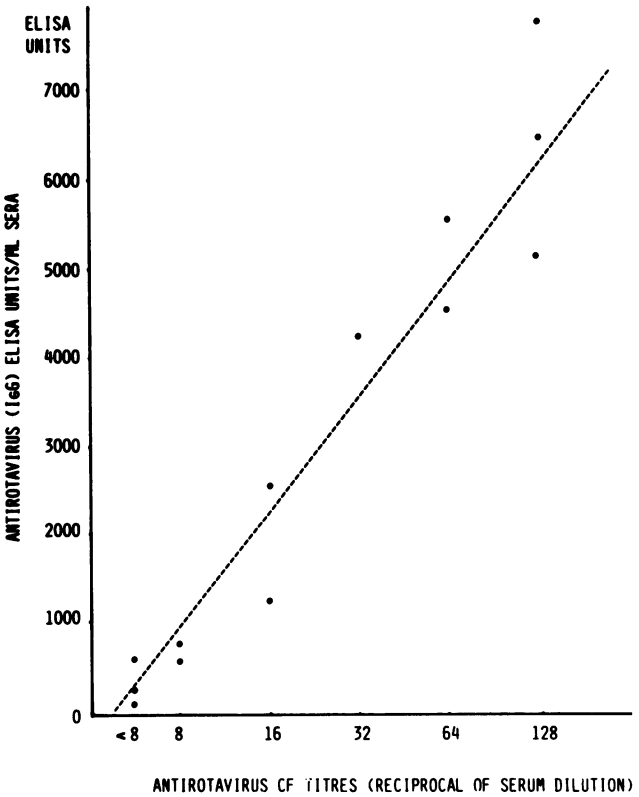

FIG. 4. Comparison of antirotavirus IgG ELISA units per milliliter with CF antirotavirus titers in 13 serum samples.

serum became an ELISA (18) with simian rotavirus (SA11) as the antigen (15). The assay depends on the fact that SA11 and human rotaviruses share a common group antigen. The test has the advantages of being more sensitive than CF in diagnosis of rotavirus infection (9) and capable of measuring antibodies of differing classes of immunoglobulin.

The rotavirus ELISA has great potential in epidemiological surveys. Changes in the amount of class specific antirotavirus IgG in serum samples from one individual can be used to detect rotavirus infection that is asymptomatic (10). However, estimation of the amount of rotavirus antibody in serum by endpoint titration is time consuming and subject to errors inherent in preparing doubling dilutions of serum samples and in determining the endpoint. The technique described in this paper proved as sensitive as CF and ELISA endpoint titration in detecting rotavirus infection. In setting up this assay, we initially chose high-titer serum from one individual as our standard serum. In future assays it would be advantageous to use a pool or sera as a standard. This would minimize errors due to the presence of antibodies of differing affinities in test serum samples, and it would increase the range of antigenic epitopes likely to be recognized by the standard serum. Use of a standard serum and inclusion of an internal control serum excludes errors in quantitation of antibody owing to day-to-day variation in background reactions in the assay. The assay was simple to perform and usually required the assay of only a single serum dilution. It has been calculated that a single determination can quantitatively measure approximately four doubling dilutions of serum (4). Our assay was reproducible, and it detected seroconversion in all of a limited selection of paired serum samples from patients with known rotavirus infections.

The standard curve method has been evaluated by de Sauvigny et al. (7), who concluded after a careful comparison of different techniques that it was the method of choice for estimation of serum antibodies to *Toxocara* spp. The standard curve method has also been applied to estimation of viral antibodies by ELISA using influenza A, cytomegalovirus, mumps virus, respiratory syncytial virus (14), herpes simplex virus (6), and the hexon antigen from adenoviruses (16) as antigens. The assay has been validated for each of these viruses and has been shown to be more sensitive than endpoint titration in detecting significant changes in antibody concentration (6). The major disadvantage of the standard curve method with the ELISA is a disadvantage that is shared by all assays based on ELISA, i.e., the fact that the technique is influenced by avidity of antibody (4, 5) and underestimates antibody of low avidity present in early specimens of convalescent-phase serum (21 to 40 days postinfection). In our experience, antibody detected in convalescent-phase serum samples obtained 21 days after a primary infection sometimes showed only a low amount of antibody. Even so, the rise in antibody detected in convalescent-phase serum samples compared with acute-phase serum samples exceeded our definition of a seroconversion, i.e., 2 × intra-run coefficient of variation of the assay. Substitution of serotypes of cultivable human rotavirus for SA11 might increase the sensitivity of the assay.

The technique described here has proved of value in a longitudinal epidemiological study of the effect of neonatal rotavirus infection on immunity to postneonatal rotavirus infection (1). By estimating the amount of rotavirus antibody in serum samples obtained at regular intervals from 106 children, we were able to detect rotavirus infections that were asymptomatic or so mild that they were attributed to other causes, e.g., teething. The assay cannot be used to estimate immune status, but it is of great value in epidemiological surveys to detect rotavirus infection. If a vaccine against rotavirus becomes available, the technique described here should assist in the assay of the serum samples generated by large-scale epidemiological studies. Adoption of this technique and distribution of a standard serum for worldwide use would facilitate comparison of results from many different laboratories.

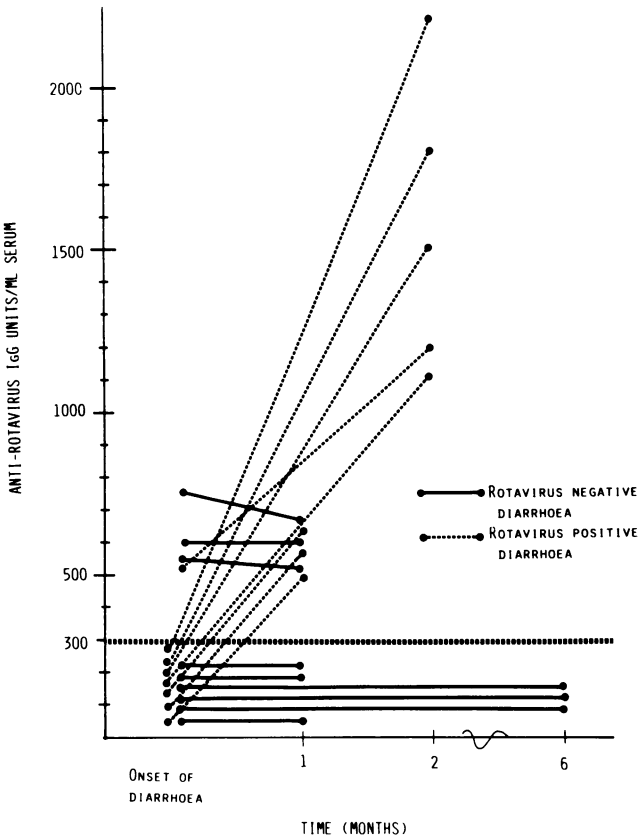

FIG. 5. Antirotavirus IgG units per milliliter in acute-phase and convalescent-phase serum samples from eight children with diarrhea due to rotavirus and nine children with diarrhea due to other causes. Horizontal line drawn at 300 IgG U/ml indicates negative-positive cutoff level. Serum samples were tested in dilutions of 1:100. Units were read from standard curves of serum A (Fig. 2) and were corrected for the dilutions in the test.

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