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Non-structural protein NSP2 induces heterotypic antibody responses during primary rotavirus infection and reinfection in children

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Abstract

Rotaviruses are the single most important causes of severe acute diarrhoea in children worldwide. Despite success in developing vaccines, there is still a lack of knowledge about many components of the immune response, particularly those to non-structural proteins. This study established radioimmunoprecipitation (RIP) assays using labelled G1P[8], G2P[4] and G4P[6] human rotaviruses to examine the spectrum and duration of rotavirus antibodies in sera collected sequentially for 18–36 months from 27 children after hospitalisation for primary rotavirus gastroenteritis. Five children experienced rotavirus re-infections. Primary responses detected to non-structural protein NSP2 declined to baseline after 100–150 days. Responses were heterotypic between NSP2 of G1P[8] and G4P[8] rotaviruses. Re-infections after 465–786 days boosted antibody levels to NSP2 of both serotypes, together with the appearance of anti-NSP2 to G2P[4], even though there was no evidence of infection with this serotype. We developed an enzyme-immunoassay to measure sequential levels of anti-NSP2 IgG and IgA, using recombinant (heterotypic) NSP2 derived from SA11 (G3P[2]). Anti-NSP2 IgG and IgA were detected in sera from 23/23 (100%) and 18/24 (75%) of children after primary infection, declined to baseline after 100–150 days, were boosted after rotavirus re-infections, and again declined to baseline 150 days later. Anti-NSP2 IgA was also detected after primary infection, in duodenal juice from 14/16 (87%), and faecal extract from 11/19 (57%) of children. Sequential estimation of anti-NSP2 EIA levels in sera could be a sensitive index of rotavirus infection and re-infection. The potential of anti-NSP2 to limit viral replication after re-infection deserves further study.

Keywords

rotavirus; NSP2; humoral immune responses; enzyme immunoassay

Introduction

Rotaviruses are a major worldwide cause of diarrhoea-associated morbidity and mortality in infants and young children, and are responsible for more than 600,000 deaths annually in

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developing countries (Parashar et al., 2006). Two recently licensed live oral rotavirus vaccines have proved very effective in prevention of severe rotavirus disease in young children in USA, Finland and Latin America (Ruiz-Palacios et al., 2006; Vesikari et al., 2006). Neither vaccine protects against rotavirus re-infection. The immunological basis for the protective immunity to severe clinical disease after re-infection is not certain (Offit, 1996; Ward, 1996).

Rotaviruses are double-stranded RNA viruses belonging to the genus *Rotavirus* in the Family *Reoviridae*. They replicate in the epithelial cells lining the small intestine of mammalian and avian species, resulting in cell cytolysis and life-threatening dehydration in some children (Estes et al., 2001). The virion has three concentric protein shells with an inner core containing three structural proteins VP1, VP2 and VP3, an inner capsid comprising the major structural protein VP6, and an outer capsid comprising the proteins VP4 and VP7. Six non-structural proteins NSP1–6 participate in replication (Estes et al., 2001). The majority of infections in children are due to Group A rotaviruses (classified on the basis of antigenic groups on VP6). Group A are further classified into serotypes based on identification of the two outer capsid protein antigens VP4 (P types) and VP7 (G types). These proteins stimulate the production of neutralising antibody *in vivo*. At least ten G types and nine P types have been identified in humans.

Studies of the natural history of rotavirus infection in children show that severe diarrhoea is usually only associated with primary infection, regardless of serotype. Rotavirus re-infections with the same or different serotypes occur regularly (perhaps annually) in young children and are often asymptomatic or associated with only mild disease (Bishop et al., 1983; Velazquez et al., 1996). This protection against severe symptoms on re-infection can be homotypic or heterotypic and is associated with development of humoral (serum and intestinal) antibodies (Bishop et al., 1990; Offit, 1996; Ward, 1996; Jiang et al., 2002) that neutralise the outer capsid proteins VP4 and VP7. These neutralising antibodies play an important, but not exclusive, role in protective immunity (Clemens et al., 1992; Ward 1996).

It is possible that antibodies to other rotavirus proteins, that appear to lack the capacity to stimulate *in vitro* neutralising antibody, could also contribute to protective immunity. RIP assays show that primary rotavirus infections are associated with rises in serum antibody levels to the structural proteins VP2, VP4, VP6, VP7, and to the non-structural proteins NSP2 and NSP4 (Svensson et al., 1987; Brussow et al., 1990; Richardson et al., 1993; Colomina et al., 1998). There is experimental evidence that antibody to VP6 can induce protective immunity in mice (Burns et al., 1996; O'Neal et al., 1997; Choi et al., 2000), possibly after transcytosis across intestinal epithelial cells followed by intracellular interference with replication (Feng et al., 2002; Schwartz-Cornil et al., 2002). Antibody to non-structural proteins could mediate protection via a similar mechanism.

There are few longitudinal studies of individual protein responses in children after primary rotavirus infection and re-infection. In particular, there are no studies in which the G- and P-types responsible for primary and secondary infections have been identified. In the present study we collected weekly faecal specimens, four-monthly sequential sera and clinical histories of diarrhoeal episodes from 27 children under surveillance for 18–36 months after hospitalisation for treatment of primary rotavirus infection. Responses to individual rotavirus proteins were assayed by RIP of sequential sera, using standard rotavirus strains representing three VP7 and three VP4 human rotavirus types. Five of these children experienced rotavirus re-infections during the period of surveillance. Re-infection was associated with visible boosts in RIP-antibody to VP7, NSP2 and NSP4. Estimation of the magnitude of responses by densitometry showed marked heterotypic responses to NSP2. We developed an enzyme immunoassay (EIA), to detect and quantitate levels of anti-NSP2 in sera and secretions, using expressed recombinant NSP2 (derived from SA11) as capture antigen. The results showed that

sequential evaluation of antibody levels to NSP2 could serve as a sensitive measure of rotavirus re-infection. The possibility that anti-NSP2 could restrict rotavirus replication after re-infection requires further study.

Methods

Subjects and serum samples

Serum and faecal samples were collected from 27 children (under 40 months of age) admitted to the Royal Children's Hospital Melbourne, Australia, with acute gastroenteritis (Table I). Ages of the children at admission ranged from 1–39 months, with 2 under 3 months of age. Rotavirus infection was diagnosed within 24 hours of admission to hospital by electron microscopy of faecal extracts. All children were shown to be experiencing a primary rotavirus infection as judged by detection of anti-rotavirus IgM in acute sera. (Grimwood et al., 1988). Infections were caused by G1P[8] in 18 children, 4 by G4P[8], 2 by mixed G1/G4 and 3 by strains that could not be typed.

Blood was initially collected within 48 hours of admission to hospital (within 7 days of disease onset). Convalescent sera was collected (after disease resolution) 29–42 days later, and thereafter at 4, 8, 12, 16, 20, 24, 28 and 32 months after admission. Blood was collected from each child by venepuncture during hospitalisation or by capillary stick method during home visits. Serum was stored in aliquots at –70°C until required. Duodenal specimens were collected from 16 children during acute and convalescent stages of the infection as described previously (Grimwood et al., 1988) and were stored at –70°C.

All children were kept under surveillance by telephone and home visits for 18–36 months after discharge. Surveillance included weekly collection of a faecal specimen and of diarrhoeal faeces as appropriate. All samples were stored in aliquots at –70°C until required. Five children experienced rotavirus re-infection (detected by EIA and/or RT-PCR) resulting in faecal excretion of G1P[8] or G1P[nt] non-typeable strains 15–26 months (465–786 days) after primary infection (Table II). Re-infection was asymptomatic (Patients 1, 10) or associated with mild diarrhoea (Patients 6, 18) or occasional vomiting lasting one day (Patient 17). Transmission of rotavirus infection within the family, as judged by coincidental symptoms of gastroenteritis in a sibling or in parents, occurred with 3 children (Patients 6, 17, 18).

Ethical approval for this study was obtained from the Human Ethics Committee of the Royal Children's Hospital. Informed consent was obtained from parents of the children prior to enrolment in the study.

Radioimmunoprecipitation assay (RIP)

Protein-specific antibody responses in sets of sera from each child were determined by radioimmunoassay (RIP) using radiolabelled standard rotavirus strains. Techniques used in the performance of the RIP assay and identification of rotavirus polypeptides reacting with patient's sera are described in detail elsewhere (Richardson et al., 1993). Briefly [³⁵S] methionine - labelled cell lysates of standard human rotavirus strains were prepared using MA104 cell cultures of RV4 (G1P[8]), RV5 (G2P[4]) and ST3 (G4P[6]). These were reacted individually with sets of sequential sera from each patient, using undiluted sera, or sera diluted 1:2 or 1:4 in PBS (pH 7.3) depending on amount of serum available. Sequential sera were not reacted with G3 strains because of insufficient quantity of sera. The complete set of serum samples from each child were assayed using identical dilutions and identical preparations of [³⁵S]-labelled rotaviruses. Immune complexes were precipitated with Pansorbin (Calbiochem), followed by solubilisation with sodium dodecyl sulfate (SDS). The immune complexes were separated by polyacrylamide gel electrophoresis and detected by fluorography. [³⁵S]-labelled

viral polypeptides were identified by their molecular weights relative to markers of known molecular weight. Results were compared with results from parallel assays of mock-infected cellular proteins.

Results of RIP assays were recorded visually and by transmission densitometry using a densitometer (Quick Scan R & D, Helena Laboratories) linked to an integrator (Quick Quant III, Helena Laboratories). Quantitative analysis of the intensity of reaction of serum with each protein involved calculation of the area beneath the peaks obtained by densitometry. This area was found to be proportionate to the intensity and width of bands visible on the autoradiograph.

Reproducibility of RIP assays and of densitometry calculations was confirmed by reacting sequential sets of sera from 11 children using different RIP preparations of RV4 or ST3. All showed the same results with each of the RIP preparations.

Expression and purification of NSP2

Gene segment 8, encoding NSP2, derived from SA11 was ligated into *Escherichia coli* M15 cells using the IPTG-inducible expression vector pQE60 as previously described (Taraporewala et al., 2004). The expressed recombinant NSP2 was purified using nickel-nitrile acetate (Ni-NTA) agarose columns according to manufacturers instructions (Qiagen, Hilden, Germany). The eluted NSP2 was dialysed in low salt buffer (2mM Tris-HCl pH7.2, 0.5M EDTA, 0.5mM dithiothreitol) for 48 hours. The concentration of the purified NSP2 was determined by Bradford assay using bovine serum albumin as the protein standard, and compared by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) according to Laemmli (1970) and Coomassie blue R-250 stained. Purified recombinant NSP2 (rNSP2) was adjusted to approximately 1mg per ml.

Enzyme-linked immunosorbent assay (EIA) of NSP2 specific antibody

Briefly, microtitre plates with high binding capacity (Nunc) were coated with 100ul/well (2ug/ml) of purified recombinant NSP2 in 0.1M carbonate-bicarbonate buffer (pH9.6), and incubated overnight at 4C. Serum samples at a single dilution (1:50 for IgA and 1:100 for IgG) were added and incubated at 37C for 1 hour for the IgG EIA, and overnight at 4C for the IgA EIA. Faecal and duodenal fluid specimens, diluted 1:50 and 1:12 in casein respectively, were incubated at 4C overnight. All dilutions of antibody and conjugates were made in PBS-Tween containing 0.5% casein. The antigen-antibody complexes were detected using horseradish-peroxidase (HRP) conjugated anti-human IgA or IgG specific antibodies (DAKO), diluted 1:800 and 1:17,500 respectively. The plates containing duodenal samples were blocked with 1% BSA in casein for 30 min at 37C. The plates were incubated at 37C for 60 min for IgG or 90 minutes for IgA. The colour reaction was developed using 3,3',5,5'-tetramethylbenzidine (TMB, Sigma) at 0.1mg/ml diluted in 0.1M Na-acetate buffer with 0.036% (v/v) H₂O₂. The reaction was stopped with 2M H₂SO₄. The optical density was measured at 450nm using an ELISA plate reader (Flow Titertek Multiskan, Flow).

All samples were assayed in duplicate against wells coated with rNSP2 antigen or carbonate buffer alone. A sample was considered positive if its optical density was equal to or greater than 0.2 and at least twice the absorbance of the background control.

Antibody titres (IgG and IgA) were expressed as units per millilitre of serum (Bishop et al., 1983). Values were determined from a standard curve of reference serum, duodenal fluid or faecal sample. To generate a standard curve, serial three fold dilutions (1:100 to 1:24,300) of an anti-rotavirus sample (known to contain specific anti-NSP2 IgG and IgA) was added to duplicate wells coated with NSP2 or carbonate buffer. The concentration of NSP2 IgG or IgA antibody in the reference sample was arbitrarily assigned a value of 20,000 units/ml as

described previously (Bishop et al., 1983). OD values from EIA assays of sequential sera and secretions were compared with values on the standard curve in order to calculate units of anti-NSP2 IgG and IgA for all test specimens. An immune conversion in serum or intestinal contents was defined as an increase in unit values (in consecutive specimens) of >2x intra-run coefficient of variation of the assay.

Reproducibility of the NSP2 EIA assay was established by testing two different dilutions of the standard positive sera 20 times each during one assay. This was repeated on 2 different days. The co-efficient of variation of rotavirus antibody unit values was calculated and ranged from 0.3 to 60.3% for IgG and 0 to 30.2% for IgA. Thus the intra-run coefficient of variation of the assay was taken as 60.3% for IgG and 30.2% for IgA.

Results

Radio-immunoprecipitation assays

i) Visual Interpretation—The results of RIP assay of primary antibody responses have been published in detail elsewhere (Richardson et al, 1993). Specific immunoprecipitation of VP2, VP4, VP6, VP7, NSP2, NSP4, and NSP5 was seen in acute and convalescent sera. Control RIP assays using aliquots of PBS in place of serum samples showed non-specific immunoprecipitation of VP1 and NSP1.

RIP results in sequential sera showed that the responses to VP7 and NSP4 detected in acute and convalescent sera declined rapidly and often were not visible by 100 days post-primary rotavirus infection. Responses to NSP2 declined progressively after primary infection, but remained detectable, even at 700 days post-primary infection. Responses to VP2, VP4 and VP6 detected in acute and convalescent sera appeared to remain steady during the course of surveillance.

Rotavirus re-infection was detected in 5 children, 15–26 months after primary infection. Ages at primary infection and re-infection, together with serotypes of infecting strains, are listed in Table II. A representative RIP result after primary rotavirus infection and re-infection (day 499) of one child (patient 1), with G1P[8] rotavirus strains is shown in Figure 1A. Figure 1B, illustrates representative RIP results after re-infection of one child (patient 2), who was re-infected with a G1P[nt] rotavirus strain 464 days after primary infection with a G1[P8] strain. Re-infection was associated with boosts of serum RIP antibody to NSP2, NSP4, and VP7 of G1, G2 and G4 strains using radiolabelled G1P[8], G2P[4] and G4P[6] strains respectively (panels A, B and C). The spectrum of responses to VP7 widened after re-infection to include responses to the VP7 protein of serotype G2 even though there was no direct evidence of infection with a G2 virus in any of the 5 children. There were no convincing changes visible in levels of RIP antibody to VP2, VP4 and VP6 of any strain.

ii) Densitometry analysis of protein bands observed in RIP assays—Densitometry measurements of sequential RIP serum antibody reactions, calculated from reactions with labelled G1P[8] rotaviruses, are graphed in Figure 2 for Patients 1, 6, 14 and 17. Sera (from the same patients) reacted with G2P[4] and G4P[6] rotaviruses showed the same densitometry patterns as seen with G1 rotaviruses (results not shown). Results from Patient 18 were not assessed by densitometry.

Each of the four patients showed post-primary decreases in reaction intensities of serum antibody with VP2, VP4 and VP6 of G1, G2 and G4 rotaviruses that had not been detected by visual inspection. Results with VP2 and VP4 are not shown in Figure 2. Marked decreases in post-primary reactions with NSP2 of each test strain were present in all four patients. After reinfection, each of the four patients showed markedly increased serum immune responses to

NSP2 of each test strain. Responses to VP6, VP7 and to NSP4 were of lesser magnitude. Responses were not consistent from child to child, possibly because of the variable intervals (ranging from 10–104 days) between date of re-infection and collection of follow-up sera. Sequential sera from 11 children not experiencing rotavirus re-infections showed no boosts in RIP densitometry calculations (results not shown).

EIA humoral immune responses to rNSP2

i) In serum, duodenal juice and faecal extracts after primary rotavirus infection

—Titres of anti-NSP2 EIA IgG units and IgA units in serum and duodenal juice from 27 children after primary infection with varying serotypes of rotavirus are listed in Table I. An anti-NSP2 IgG seroconversion was detected in all 23 children assayed, including three infants aged 3 months or less. An anti-NSP2 EIA IgA seroconversion was detected in 18/24 (75%) of children. An IgA response in antibody levels to NSP2 was detected in paired specimens of duodenal juice from 14/16 (87%) children, and in faeces from 11/19 (57%) of children (results not given).

ii) In sequential sera—Levels of EIA anti-NSP2 IgG and IgA in sequential sera specimens collected from 11 patients with no evidence of later re-infection, peaked in convalescent sera taken 24 – 40 days after primary rotavirus infection, followed by a decline to baseline levels after 100 – 150 days. None showed changes in anti-NSP2 antibodies during further surveillance for 18–36 months (results not shown). Representative anti-NSP2 levels in one child over 712 days (Patient 12) are illustrated in Figure 3A.

Sequential serum anti-NSP2 EIA levels were analysed in 4 of the 5 children with rotavirus re-infections. Three showed an anti-NSP2 EIA IgG seroconversion after re-infection (Patients 6, 17, 18). The fourth child (Patient 1) showed a rise of approximately 100% in anti-NSP2 IgG that did not meet the criterion for seroconversion. Follow-up serum from this child had been collected approximately 100 days after detection of rotavirus re-infection. Figure 3B illustrates the results from one of the children (Patient 6). The decline in anti-NSP2 IgG antibody levels after primary infection was followed by a marked boost in anti-NSP2 after re-infection, followed by a second decline.

Two of the five children under long-term surveillance had a second seroconversion detected by a rise in anti-NSP2 EIA IgG antibody levels from baseline to >3,500 units. These seroconversions were associated temporally with mild vomiting and diarrhoea. Rotavirus re-infections could not be confirmed by EIA or RT-PCR of faecal extracts (data not shown).

Discussion

This study is unique in its examination of longitudinal protein-specific serum antibody levels in 27 children during 36 months after hospitalisation with primary rotavirus infection. Rotavirus re-infections (asymptomatic or associated with mild diarrhoea) were identified in 5 children 15–26 months after primary infection. During the three years of surveillance, persisting levels of antibody to VP2, VP4 and VP6 were readily detected by visual inspection of gels representing RIP assays using labelled rotavirus serotypes G1P[8], G2P[4] and G4P [6]. However responses to VP7, NSP2 and NSP4 decreased markedly by approximately 100 days after primary infection, and remained low, until re-infection of five children boosted RIP serum immune responses to the rotavirus proteins VP7, NSP2, and NSP4 of all three serotypes. Measurement of these responses by densitometry detected increases in anti-VP6 not detected by eye, in addition to showing that antibody responses to NSP2 were of greater magnitude than the responses to the other proteins. Heterotypic antibody to the proteins of G1 and G4 rotavirus serotypes (including VP7) were detected after primary infection with G1 or G4. In addition, antibody to proteins of G2 rotavirus were detected after re-infection, even though there was

no evidence of infection of these children with G2 serotypes. The results confirm previous results showing broadening of the neutralising antibody responses using single (VP7) gene reassortants (Gorrell and Bishop, 1999)

We developed a simple, sensitive and specific enzyme immunoassay to quantitate anti-NSP2 responses in human sera and secretions after rotavirus infections using capture antigen derived by cloning and expression of NSP2 of SA11. An anti-NSP2 IgG seroconversion was detected in all children admitted to hospital with acute primary rotavirus infection, associated with anti-NSP2 IgA seroconversion in 71% of the same children. Anti-NSP2 IgA antibody responses were also identified in duodenal fluid of 87% of children and in faeces of 47% of children. After primary infection, the high anti-NSP2 serum antibody levels reverted to baseline within 100 – 150 days post infection. Rotavirus re-infection in four children, 15–26 months after primary infection, was again associated with anti-NSP2 immune responses in serum. Quantitation of EIA serum anti-NSP2 IgG was a sensitive measure of primary rotavirus infection, even in infants infected during the first three months of life. Immune responses directed toward NSP2 were heterotypic across VP7 G serotypes. RIP assays using labelled G1P[8], G2P[4] and G4P[6] serotypes detected rises in serum antibody to the VP7 of each serotype, even though most of the children had not experienced infections with the latter two serotypes. In addition, the NSP2 protein used in this study, derived from SA11 (G3P[1]), detected anti-NSP2 antibody in sera and secretions from children infected with serotype G1P[8] or G4P[8] rotavirus strains. Taken together, these results suggest that NSP2 has conserved immunoreactive regions present in many rotavirus serotypes. Unpublished results obtained recently in this laboratory has revealed a high level of conservation in genes coding for NSP2 (N Donker and C Kirkwood, unpublished observations) Characterisation of the immunological active epitopes is required to further determine the conservation of the epitope and resultant protein.

There is only limited information about the role of other rotavirus non-structural proteins in stimulating an immune response following natural infection. Antibody seroconversions to the rotavirus NSP4 protein have been identified in 54–55% in children with natural rotavirus infection (Ray et al., 2003; Vizzi et al., 2005). In contrast, our study identified a seroconversion rate to NSP2 of 100% in children with primary rotavirus infection. It is interesting to speculate why the NSP2 and NSP4 proteins appear to differ in their ability to induce antibody responses after natural infection. As suggested by Vizzi et al. (2005), the NSP4 protein may be ineffective in epitope exposure and presentation to the immune system. Our results imply that the NSP2 protein is highly immunogenic.

The results of this study suggest that antibody to the non-structural protein NSP2 in addition to being a sensitive measure of rotavirus infections, might also contribute to the development of the clinical protection against disease symptoms observed after re-infection (Bishop et al., 1983; Velazquez et al., 1996). There is convincing evidence that protection after re-infection involves components of the memory immune response that limit rotavirus replication (and consequent damage to the small intestine). A marked reduction in excretion of rotavirus particles has been repeatedly noted after rotavirus re-infection in children, and together with immunological measures is regarded as a measure of protective efficacy in humans and in animal models (Ward, 1996). Significant effort however has been unable to prove the exclusive involvement of antibodies to rotavirus structural proteins (VP7, VP4 and VP6) in protection. The mechanism underlying this reduced excretion is not clear. Neutralising antibodies to the rotavirus outer capsid proteins VP4 and VP7 can limit viral replication by external interference with cell binding and penetration. Intracellular antibodies reacting with the structural protein VP6 via transcytosis of antibody have been shown to interfere with viral replication and could contribute to protection (Choi et al., 2000; Feng et al., 2002; Schwartz-Cornil et al., 2002). It is possible that antibody to non-structural proteins, in particular NSP2, could interfere with

early assembly of translated structural proteins into viroplasms by an analogous mechanism involving transcytosis. NSP2 is essential for forming viral factories that support genome replication and packaging, viral transcription and core and double layered particle assembly (Taraporewala et al. 1999;2002; Taraporewala and Patton, 2004), and the presence of NSP2 antibodies intracellularly could have a cascade effect on numerous downstream events in the viral life cycle. The inhibition of rotavirus replication by NSP2-specific siRNA supports this role (Silvestri et al., 2004). The potential inhibitory property of these antibodies is supported by preliminary *in vitro* evidence that antibody against NSP2 can limit rotavirus replication (C. Kirkwood, unpublished observations). The possibility that anti-NSP2 could restrict rotavirus *in vivo* requires further study. Evidence in support of this hypothesis could have implications for further vaccine development, particularly as components for inclusion in parenterally administered vaccines.

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References

- Bishop RF, Barnes GL, Cipriani E, Lund JS. Clinical immunity after neonatal rotavirus infection. A prospective longitudinal study in young children. *N Engl J Med* 1983;309:72–76. [PubMed: 6304516]
- Bishop, R.; Lund, J.; Cipriani, E.; Unicomb, L.; Barnes, G. Clinical serological and intestinal immune responses to rotavirus infection of humans. In: de la Maza, LM.; Peterson, EM., editors. *Medical Virology*. Vol. 9. New York: Plenum press; 1990. p. 85-110.
- Burns JW, Siadat-Pajouh M, Krishnaney AA, Greenberg HB. Protective effect of rotavirus VP6-specific IgA monoclonal antibodies that lack neutralizing activity. *Science* 1996;272:104–107. [PubMed: 8600516]
- Brussow H, Offit PA, Gerna G, Bruttin A, Sidoti J. Polypeptide specificity of antiviral serum antibodies in children naturally infected with human rotavirus. *J Virol* 1990;64:4130–4136. [PubMed: 2166808]
- Choi AH, Basu M, McNeal MM, Flint J, VanCott JL, Clements JD, Ward RL. Functional mapping of protective domains and epitopes in the rotavirus VP6 protein. *J Virol* 2000;74:11574–11580. [PubMed: 11090155]
- Clemens JD, Ward RL, Rao MR, Sack DA, Knowlton DR, van Loon FP, Huda S, McNeal M, Ahmed F, Schiff G. Seroepidemiologic evaluation of antibodies to rotavirus as correlates of the risk of clinically significant rotavirus diarrhea in rural Bangladesh. *J Infect Dis* 1992;165:161–165. [PubMed: 1309372]
- Colomina J, Gil MT, Codoner P, Buesa J. Viral proteins VP2, VP6, and NSP2 are strongly precipitated by serum and fecal antibodies from children with rotavirus symptomatic infection. *J Med Virol* 1998;56:58–65. [PubMed: 9700634]
- Estes MK, Kang G, Zeng CQ, Crawford SE, Ciarlet M. Pathogenesis of rotavirus gastroenteritis. *Novartis Found Symp* 2001;238:82–96. [PubMed: 11444037]discussion 96–100.
- Feng N, Lawton JA, Gilbert J, Kuklin N, Vo P, Prasad BV, Greenberg HB. Inhibition of rotavirus replication by a non-neutralizing, rotavirus VP6-specific IgA mAb. *J Clin Invest* 2002;109:1203–1213. [PubMed: 11994409]
- Gorrell RJ, Bishop RF. Homotypic and heterotypic serum neutralizing antibody response to rotavirus proteins following natural primary infection and re-infection in children. *J Med Virol* 1999;57:204–211. [PubMed: 9892409]
- Grimwood K, Lund JC, Coulson BS, Hudson IL, Bishop RF, Barnes GL. Comparison of serum and mucosal antibody responses following severe acute rotavirus gastroenteritis in young children. *J Clin Microbiol* 1988;26:732–738. [PubMed: 2835391]

- Jiang B, Gentsch JR, Glass RI. The role of serum antibodies in the protection against rotavirus disease: an overview. *Clin Infect Dis* 2002;34:1351–1361. [PubMed: 11981731]
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685. [PubMed: 5432063]
- O'Neal CM, Crawford SE, Estes MK, Conner ME. Rotavirus virus-like particles administered mucosally induce protective immunity. *J Virol* 1997;71:8707–8717. [PubMed: 9343229]
- Offit PA. Host factors associated with protection against rotavirus disease: the skies are clearing. *J Infect Dis* 1996;174(Suppl 1):S59–S64. [PubMed: 8752292]
- Parashar UD, Gibson CJ, Bresee JS, Glass RI. Rotavirus and severe childhood diarrhea. *Emerg Infect Dis* 2006;12:304–306. [PubMed: 16494759]
- Ray P, Malik J, Singh RK, Bhatnagar S, Bahl R, Kumar R, Bhan MK. Rotavirus nonstructural protein NSP4 induces heterotypic antibody responses during natural infection in children. *J Infect Dis* 2003;187:1786–1793. [PubMed: 12751037]
- Richardson SC, Bishop RF. Homotypic serum antibody responses to rotavirus proteins following primary infection of young children with serotype 1 rotavirus. *J Clin Microbiol* 1990;28:1891–1897. [PubMed: 2172292]
- Richardson SC, Grimwood K, Bishop RF. Analysis of homotypic and heterotypic serum immune responses to rotavirus proteins following primary rotavirus infection by using the radioimmunoprecipitation technique. *J Clin Microbiol* 1993;31:377–385. [PubMed: 8381813]
- Ruiz-Palacios GM, Perez-Schael I, Velazquez FR, Abate H, Breuer T, Clemens SC, Chevart B, Espinoza F, Gillard P, Innis BL, Cervantes Y, Linhares AC, Lopez P, Macias-Parra M, Ortega-Barria E, Richardson V, Rivera-Medina DM, Rivera L, Salinas B, Pavia-Ruz N, Salmeron J, Ruttimann R, Tinoco JC, Rubio P, Nunez E, Guerrero ML, Yarzabal JP, Damaso S, Tornieporth N, Saez-Llorens X, Vergara RF, Vesikari T, Bouckennooghe A, Clemens R, De Vos B, O'Ryan M. Human Rotavirus Vaccine Study Group. Safety and efficacy of an attenuated vaccine against severe rotavirus gastroenteritis. *N Engl J Med* 2006;354:11–22. [PubMed: 16394298]
- Schwartz-Cornil I, Benureau Y, Greenberg H, Hendrickson BA, Cohen J. Heterologous protection induced by the inner capsid proteins of rotavirus requires transcytosis of mucosal immunoglobulins. *J Virol* 2002;76:8110–8117. [PubMed: 12134016]
- Silvestri LS, Taraporela ZF, Patton JT. Rotavirus replication: plus sense templates for double stranded RNA synthesis are made in viroplasm. *J Virol* 2004;78:7763–7774. [PubMed: 15220450]
- Svensson L, Sheshberadaran H, Vene S, Norrby E, Grandien M, Waddell G. Serum antibody responses to individual viral polypeptides in human rotavirus infections. *J Gen Virol* 1987;68:643–651. [PubMed: 3029295]
- Taraporewala Z, Chen D, Patton JT. Multimers formed by the rotavirus nonstructural protein NSP2 bind to RNA and have nucleoside triphosphatase activity. *J Virol* 1999;73:9934–9943. [PubMed: 10559306]
- Taraporewala ZF, Patton JT. Nonstructural proteins involved in genome packaging and replication of rotaviruses and other members of the Reoviridae. *Virus Res* 2004;101:57–66. [PubMed: 15010217]
- Taraporewala ZF, Schuck P, Ramig RF, Silvestri L, Patton JT. Analysis of a temperature-sensitive mutant rotavirus indicates that NSP2 octamers are the functional form of the protein. *J Virol* 2002;76:7082–7093. [PubMed: 12072508]
- Velazquez FR, Matson DO, Calva JJ, Guerrero L, Morrow AL, Carter-Campbell S, Glass RI, Estes MK, Pickering LK, Ruiz-Palacios GM. Rotavirus infections in infants as protection against subsequent infections. *N Engl J Med* 1996;335:1022–1028. [PubMed: 8793926]
- Vesikari T, Matson DO, Dennehy P, Van Damme P, Santosham M, Rodriguez Z, Dallas MJ, Heyse JF, Goveia MG, Black SB, Shinefield HR, Christie CD, Ylitalo S, Itzler RF, Coia ML, Onorato MT, Adeyi BA, Marshall GS, Gothefors L, Campens D, Karvonen A, Watt JP, O'Brien KL, DiNubile MJ, Clark HF, Boslego JW, Offit PA, Heaton PM. Rotavirus Efficacy and Safety Trial (REST) Study Team. Safety and efficacy of a pentavalent human-bovine (WC3) reassortant rotavirus vaccine. *N Engl J Med* 2006;354:23–33. [PubMed: 16394299]
- Vizzi E, Calvino E, Gonzalez R, Perez-Schael I, Ciarlet M, Kang G, Estes MK, Liprandi F, Ludert JE. Evaluation of serum antibody responses against the rotavirus nonstructural protein NSP4 in children

after rhesus rotavirus tetravalent vaccination or natural infection. Clin Diagn Lab Immunol 2005;12:1157–1163. [PubMed: 16210477]

Ward RL. Mechanisms of protection against rotavirus in humans and mice. J Infect Dis 1996;174(Suppl 1):S51–S58. [PubMed: 8752291]

Figure 1.

SDS- PAGE analysis of [35S] methionine-labelled rotavirus proteins immunoprecipitated with serum collected from patients who experienced a serotype G1P[8] primary and secondary rotavirus infections. Figure 1A shows the acute, convalescent and follow-up sera samples from patient 1, while Figure 1B shows the follow-up sera from patient 2. The arrows represent rotavirus during re-infection. In Figure 1B, serotype G1P[8] strain RV4 was used in panel (a), serotype G4P[6] strain ST3 was used in panel (b), and serotype G2P[4] strain RV5 was used in panel (c). Serum was used undiluted in this assay, and day of collection is indicated at bottom of each lane. Positions of rotavirus proteins (indicated in the left margin) were estimated by comparing migration relative to those of standard proteins of known molecular weight in a 10% polyacrylamide gel. The bands visible above VP2 and under NSP2 are non-specific reactions.

↑ = rotavirus re-infection (G1P[8])

** = non-specific reactions

Figure 2.

Scans of X-ray films of RIP assays using transmittance densitometry to measure immune responses to rotavirus proteins VP6, VP7, NSP2 and NSP4 of G1P[8] rotavirus in consecutive serum samples from Patients 1, 6, 17 and 18. Identity of primary rotavirus infections and re-infections (detected by EIA or RT-PCR of faecal specimens) are indicated. Vertical axis shows densitometer units calculated as a measure of both band intensity and width.

↑ = rotavirus re-infection

primary G1
rotavirus infection

Panel A. Primary rotavirus infection and associated anti-NSP2 IgG serum response of patient 12.

primary G1
rotavirus infection

secondary G1
rotavirus infection (d464)

Panel B. Primary and secondary rotavirus infections and associated anti-NSP2 IgG serum response of patient 6.

Figure 3.

Panel A. Longitudinal serum IgG antibody units to NSP2 in a child with primary rotavirus serotype G1 infection only (Patient 12, Table 1). Panel B. Longitudinal serum IgG antibody units to NSP2 in a child with primary G1 and secondary G1 rotavirus infections (Patient 6).

Table 1
IgG and IgA antibody titres to NSP2 in serum and duodenal contents from children admitted to hospital with primary rotavirus infection.

Patient	G type (age in months)	IgG units		serum IgA units		duodenal contents IgA	
		Ac	Con	Ac	Con	Ac	Con
1* (3)	1	<500	1,200	700	600	5700	24,300
2 (39)	1	<500	7,500	<300	2,150	17,300	1,900
3 (23)	1	nd	nd	<300	13,200	900	17,800
4 (14)	1	<500	11,100	1,650	7,200	1,500	7,000
5 (11)	1	<500	2,200	<300	3,550	900	2,000
6* (2)	1	<500	2,000	<300	2,100	9300	21,900
7 (20)	4	nd	nd	<300	<300	1,600	700
8 (10)	1	<500	4,300	<300	650	500	11,100
9 (16)	1	500	5,700	<300	<300	19,500	29,200
10* (20)	1	<500	4,900	<300	800	31,700	1,700
11 (21)	1/4	<500	8,000	<300	2,500	3,000	22,200
12 (21)	1	<500	6,700	<300	4,050	9,900	9,500
13 (28)	1	<500	5,900	<300	400	2,100	44,400
14 (15)	1	<500	6,500	<300	2,650	5,000	34,400
15 (27)	1	1,300	10,800	350	2,900	4,900	22,300
16 (11)	nd	nd	nd	300	300	2,200	5,700
17* (17)	4	500	4,900	nd	nd	nd	nd
18* (17)	1	<500	7,500	nd	nd	nd	nd
19 (14)	nd	<500	9,600	nd	nd	nd	nd
20 (10)	nd	<500	2,000	<300	<300	nd	nd
21 (1)	1	<500	4,000	<300	10,850	nd	nd
22 (24)	4	<500	1,700	<300	<300	nd	nd
23 (21)	4	<500	28,300	<300	<300	nd	nd
24 (6)	1	<500	4,500	1,750	600	nd	nd
25 (23)	1	nd	nd	<300	10,600	nd	nd
26 (25)	1	<500	5,300	300	1,200	nd	nd

Patient	G type (age in months)	IgG units		serum IgA units		duodenal contents IgA	
		Ac	Con	Ac	Con	Ac	Con
27 (18)	1/4	<500	6,400	<300	600	nd	nd

Convalescent sera and duodenal contents were collected between days 29-42 post rotavirus infection.

Ac = acute infection

Con = convalescent infection

IgG values of _ 500 are considered positive.

IgA values of ≥ 300 are considered positive.

Duodenal content values of ≥500 are considered positive

* = children with rotavirus re-infection

nd = not determined

Table II
Rotavirus anti-NSP2 IgG antibody titres in sequential sera from children after primary infections and re-infections.

Patient	Primary Infection		Re-infection		Serum - anti-NSP2 IgG units				re-infection	
	age (mon)	type	age (mon)	type	Ac	Con	4 month	pre	post	samples
1	3	G1P[8]	19	G1P[8]	<500	1,200	<500	<500	700	<500
6	2	G1P[8]	17	G1P[nt]	<500	2,000	<500	<500	2,200	600
10	20	G1P[8]	45	G1P[8]	<500	4,900	800	<500	4,200	na
17	17	G4P[nt]	43	G1P[nt]	500	4,900	1,700	na	na	na
18	17	G1P[8]	33	G1P[nt]	<500	7,500	700	600	8,000	1,000

nt = non-typeable by EIA or RT-PCR
na = sera not available or insufficient quantity
IgG values of ≥ 500 are considered positive
Ac = acute infection
Con = convalescent infection