

Preponderance of IgM from blood lymphocytes in response to infantile rotavirus gastroenteritis

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Abstract

Immune responses triggered by acute rotavirus infection in infants are poorly defined. To obtain indirect evidence as to gut immune response to rotavirus, the solid phase enzyme linked immunoassay (ELISPOT) of immunoglobulin and specific antibody secreting cells among circulating blood lymphocytes was used. Seventeen well nourished patients from seven to 25 months of age were studied during the peak of rotavirus infection, and in convalescence. A transient but distinct immunoglobulin secreting cell response in IgM and IgG, but not in IgA, classes was found during the diarrhoeal phase. This response included a quantitatively prominent activation of specific antibody secreting cells against rotavirus in the IgM class, mean (95% confidence interval (CI)) $82 (32, 210) / 10^6$ cells *v* $8 (4, 17) / 10^6$ cells in convalescence, $p=0.01$. The response in the IgA class was not significant. The results indicate that local immune mechanisms are activated in rotavirus diarrhoea. They further suggest that although IgA is the predominant immunoglobulin operative in the gastrointestinal tract, this may not be the case in infantile rotavirus enteritis.

Rotaviruses have emerged as a major cause of acute enteritis in infants and small children worldwide, and the most vulnerable age group is from six to 24 months.^{1,2} Symptoms include vomiting, fever, and watery diarrhoea, resulting in dehydration. In most cases the disease in our society is self limiting. Rotavirus infection may also have a long lasting effect evolving into chronic diarrhoea.³ Rotavirus destroys intestinal enterocytes in a patchy fashion, thus causing partial malabsorption.⁴ Mucosal permeability to antigens is increased,⁵ which may lead to food intolerance if local protective mechanisms fail. The factors determining the outcome remain largely unknown, but local intestinal immunity may play a major part in host resistance. Based on this, every effort is being made to develop an effective oral vaccine against rotavirus,^{1,6} underlining the importance of elucidating the immune responses evoked by it.

To study the local immune response evoked by rotavirus, a new immunoassay, ELISPOT, was used.^{7,8} The intestinal immune response is measured indirectly by detecting immunoglobulin and specific antibody secreting cells among blood lymphocytes. During infection lymphocytes are primed by antigens in the intestinal mucosa and thereafter circulate in the peripheral blood to mature. These lymphocytes then localise in the intestine to secrete antibodies against the priming antigen. Their numbers have

previously been shown to be maximal one week after peroral antigen administration.⁷

Methods

PATIENTS AND MANAGEMENT

Twenty one well nourished children (age range seven to 25 months) admitted for acute gastroenteritis of a mean (SD) $3.1 (1.1)$ days' duration, at the Department of Pediatrics, Tampere University Hospital, were enrolled in the present study. The study was carried out between January and May 1990, corresponding to the rotavirus epidemic season in Finland. Rotavirus was the aetiology in 17 cases, who thus comprised the actual study group, with a mean (SD) age of $14.3 (4.4)$ months. One patient also had a respiratory infection, treated with antibiotics, and *Clostridium difficile* was later grown from his faeces. In one additional case the diarrhoea was caused by *Salmonella typhi*.

Informed consent was obtained from the patients' parents and the study was approved by the Hospital's Committee on Ethical Practice.

Upon admission, the children were weighed and clinically examined. They were treated according to the current practice of oral rehydration and rapid refeeding appropriate for age.⁹ Weight was checked daily. The quality and number of stools (characterised as watery, loose, or solid) were followed by the attending nurses. The attending physician decided on discharge of the patients according to their clinical condition. They were seen three weeks later in convalescence or if diarrhoea recurred.

SAMPLES

Rotavirus antigen in faeces was tested with an enzyme immunoassay (Rotazyme®, Abbott Laboratories). Faeces were cultured for *salmonella*, *shigella*, *campylobacter* and *yersinia*.

Blood in heparin was drawn for the ELISPOT assay on the day after admission and at convalescence. We were unable to obtain three samples in the acute and five samples in the convalescent phase, and in one case only the number of specific antibody secreting cells could be determined from the amount of blood available.

DETERMINATION OF THE IMMUNE RESPONSE

The ELISPOT method was used, as previously described.^{7,10} The total number of immunoglobulin secreting cells was measured apart from that of specific antibody secreting cells directed against rotavirus. In brief, mononuclear cells containing mainly lymphocytes were obtained

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TABLE Clinical characteristics on admission of patients hospitalised for acute rotavirus diarrhoea (n=17)

	Mean	(SD)
Age (months)	14.3	(4.4)
Weight (g)	9660	(1990)
Dehydration (%)	5.0	(2.8)
Base excess (mmol/l)	-7.2	(3.2)
Serum sodium (mol/l)	138	(4)

by Ficoll-Paque® (Pharmacia, Sweden) centrifugation of heparinised blood. Isolated cells, washed three times in Hank's buffered salt solution (Flow Laboratories, Irvine, Scotland), were suspended in culture medium and adjusted to a concentration of 2×10^6 cells/ml.

Cells were incubated in antigen coated flat bottomed 96 well microtitre plates (Immuno-plate R I, A/S Nunc, Roskilde, Denmark). For determination of immunoglobulin secreting cells the wells were coated with rabbit antihuman IgA (α -chain) and IgM (μ -chain) (Dakopatts a/s, Roskilde, Denmark) and goat antihuman IgG (gamma chain specific) F(ab')₂ fragment of antibody (Sigma Chemical Co, St Louis, MO, USA). To determine the number of specific antibody secreting cells against rotavirus, the coating was done in two stages. First, the wells were coated for two hours at 37°C or overnight at 4°C with rabbit antirotavirus (human) (Dakopatts a/s, Glostrup, Denmark). After washing the wells with phosphate buffered saline 0.05% Tween 20, rotavirus antigen (for the complement fixation test, Behringwerke AB, Marburg, Germany) was applied for two hours at 37°C. This two stage coating was found superior to mere rotavirus antigen coating in preliminary ELISA tests on rotavirus positive and negative sera.

The antibodies secreted by the cells were visualised by application of enzyme labelled antisera, goat antihuman IgG (gamma-chain, F(ab')₂ fragment), IgA (α -chain) and IgM (μ -chain), all in alkaline phosphatase conjugate (Sigma Chemical Co, St Louis, MO, USA),

followed by a substrate agarose overlay and observation of the coloured spots.

STATISTICAL ANALYSES

Because of skewed distributions of immunoglobulin secreting cell numbers, logarithmic transformation was used and the numbers are given as geometric mean (95% confidence interval (CI)). Paired t test was used for comparing repeated measurements. χ^2 test was applied to determine differences in proportions. Spearman's rank correlation coefficient was applied to determine the significance of association between age and ELISPOT response.

Results

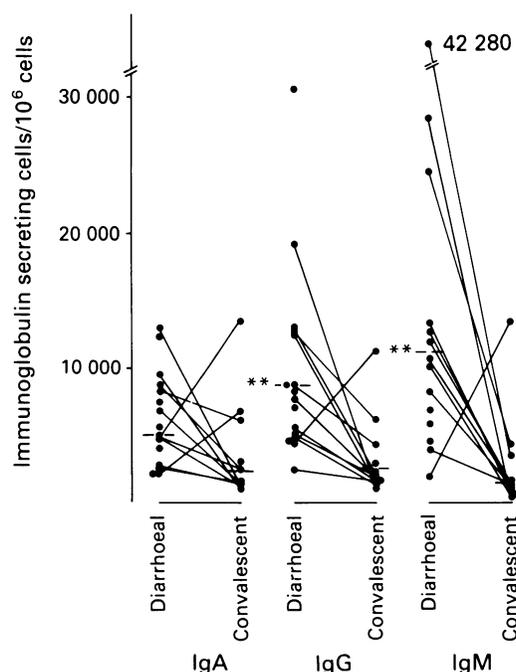
CLINICAL OUTCOME

The clinical characteristics on admission are featured in the Table. During the first day at the hospital, 94% of the patients passed watery stools. The rehydration and rapid realimentation resulted in weight gain: mean (95% CI) 275 g (167, 454). After admission, the mean (SD) duration of diarrhoea was 2.5 (1.4) days. The patients recovered uneventfully and during follow up there were no recurrences of diarrhoea.

ANTIGEN NON-SPECIFIC IMMUNE RESPONSE, IMMUNOGLOBULIN SECRETING CELLS

There was a strong IgM immunoglobulin secreting cell response during the diarrhoeal phase of the infection: mean (95% CI) 11 316 (7369, 17 379) / 10^6 cells, to 1485 (751, 2934) / 10^6 cells in the convalescent phase (Figure). There was no correlation between the magnitude of IgM immunoglobulin secreting cell response and the age of the patient ($\rho=0.02$, $p=0.90$). A similar response of lesser magnitude was seen in IgG immunoglobulin secreting cells: 8734 (6235, 12 234) to 2527 (1572, 4064) / 10^6 cells. The IgA immunoglobulin secreting cell response during diarrhoea was of a low magnitude: 5004 (3502, 7151) to 2421 (1353, 4333) / 10^6 cells. The mean difference (95% CI) in the total number of immunoglobulin secreting cells was statistically significant in the IgM and IgG class; 13 300 (4740, 21 861) / 10^6 cells and 8091 (1802, 14 380) / 10^6 cells, respectively, whereas that in the IgA class, 3070 (-1055, 7194) / 10^6 cells, was statistically not significant.

A decreasing trend can be seen from the acute to the convalescent phase in the numbers of immunoglobulin secreting cells in all isotypes (Figure). One (16.5 months) child showed a reverse pattern in all Ig classes, but cannot be distinguished from the other patients by the outcome or the severity of the infection. The second (19.5 months) child to react against the trend, but in IgA immunoglobulin secreting cells only, had clostridial overgrowth in the faeces. The one patient (18 months) with diarrhoea caused by *Salmonella typhi* responded as follows (acute to convalescent): immunoglobulin secreting cells IgM 3913 to 1580 / 10^6 cells, IgG 2387 to 1600 / 10^6 cells and IgA 8754 to 2540 / 10^6 cells.



Number of immunoglobulin secreting cells (ISC)/ 10^6 cells during the acute, diarrhoeal, n=14, and convalescent, n=11, phase of rotavirus infection. Values obtained for individual patients depicted as points, interconnected if both acute and convalescent available. Geometric means shown with horizontal lines. **denotes $p<0.01$, acute v convalescent.

SPECIFIC IMMUNE RESPONSE AGAINST ROTAVIRUS, SPECIFIC ANTIBODY SECRETING CELLS

Coinciding with the immunoglobulin secreting cell response, also the specific antibody secreting cell response in the IgM class quantitatively clearly outnumbered the response in both the IgG and IgA classes. All (100%) rotavirus positive patients showed specific antibody secreting cells in the IgM class; mean (95% CI) 82 (32, 209) /10⁶ cells during diarrhoea to 8 (4, 20) /10⁶ cells at convalescence. In 13 of 14 (93%) patients an IgG specific antibody secreting cell response was seen: three (0.6, 14) to four (0.2, 54) /10⁶ cells. An IgA specific antibody secreting cell response was seen in only nine of the 14 patients (64%), and it was small and inconsistent: 0.2 (0.02, 2.7) to 0.08 (0.004, 2) /10⁶ cells.

Discussion

Our data show that intestinal immune mechanisms are activated in acute rotavirus infection. The ELISPOT assay provides the means of indirectly measuring this response in the peripheral blood. Studying the local immune response of the intestine is complicated even with more invasive methods such as biopsy or duodenal fluid sampling,¹¹ and especially so in infants with diarrhoea. The ELISPOT assay is based on the maturation cycle of gut associated lymphoid tissue derived lymphocytes through peripheral blood back to the intestine. As well as experimental animals, it has been shown in man that the number of these cells in the blood reaches a maximum six to eight days after antigen administration, several days before the antibody response can be detected.^{8,12,13} In addition, it has been shown that the lymphocytes can be more consistently measured in blood than the antibody response in serum or duodenal fluid.⁸ The ELISPOT response wanes one week after antigen exposure is discontinued, but prolonged exposure leads to a prolonged response.^{13,14}

In this study the local immune response to rotavirus was characterised by measuring the antigen non-specific and antigen specific response in peripheral blood. Shedding of rotavirus lasts only for five to seven days,¹⁵ whereafter the antigenic drive to induce lymphocytes on their maturation cycle ceases; this might explain the clearly decreased immune response in peripheral blood in convalescence. The antigen non-specific immunoglobulin secreting cell response could be polyclonal, possibly directed against gut microbial flora¹⁰ and dietary antigens. It may be important in controlling the penetration of such antigens through the damaged mucosa and also in preventing acute spread of the infection. Conversely, the specific antibody secreting cell response is specifically directed against the infectious agent and may be vital in preventing the prolongation of the diarrhoea and in protection against reinfection.

We have previously shown a strong IgA immunoglobulin secreting cell response one week after infection during acute bacterial diarrhoea in adults.¹⁰ We have shown that the specific antibody secreting cell response during acute bacterial diarrhoea and to *Salmonella typhi* (Ty21a) vaccine is also predominantly in the IgA

class in adults.^{7,10} The predominance of IgA in adults has been confirmed by others, showing IgA, IgG, but no IgM secreting cells after oral cholera vaccination.¹³ In this study, corresponding antigen non-specific and specific immune responses were shown in children with rotavirus diarrhoea, but in contrast with the IgA-dominated adult response, the children reacted with IgM.

Rotavirus infection seriously challenges the integrity of the intestinal barrier functions and there is a need for instant local defence. It is conceivable that the IgM secreting cell response can be escalated more rapidly than the IgA secreting cell response. Supporting this suggestion an IgM response also precedes the IgA response to intestinal bacteria as measured in stool samples of neonates.¹⁶ An early IgM response has been shown in the duodenal fluid during acute enteropathogenic *Escherichia coli* diarrhoea in children.¹⁷ In duodenal fluid, rotavirus specific IgM antibodies have been shown to be raised one week after the onset of symptoms,¹¹ corresponding to our finding of high numbers of IgM secreting cells in the blood a few days earlier. It has been shown that in duodenal fluid IgA dominates four to five weeks after rotavirus infection.¹¹ A shift from IgM to IgA may be effected locally,¹⁸ which could not be measured by the ELISPOT. This would, however, not explain the demonstrated difference between the immune response of adults and infants.

The unexpected dominance of IgM in the immune response may be the result of either antigen or host dependent factors. The quality of the immune response may therefore be explained by the infectious agent, viral or bacterial. This, in turn, may be connected with the properties of their antigenic determinants, mainly proteins on viruses and lipopolysaccharides and other macromolecules on bacteria. In support of this the immunoglobulin secreting cell response of the one child with *Salmonella* enteritis was mostly IgA, as was the response of the child with additional clostridial overgrowth.

It is tempting to speculate that in early childhood IgM compensates for the relative immaturity of the intestinal IgA system. Neonatal B lymphocytes have been shown functionally defective in their capacity to generate Ig-producing cells in vitro compared with adult B cells, and this immaturity is reflected particularly in the IgA response.¹⁹ Healthy neonates are able to produce secretory IgA antibodies,¹⁶ but the levels remain at adult lower normal limit until the age of 12 months.²⁰ The relative immaturity of the intestinal IgA system may thus only become manifest at times of greater than normal challenges to the intestine, such as in diarrhoea caused by rotavirus.

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