Systemic immune response after rotavirus inoculation of neonatal mice depends on source and level of purification of the virus: implications for the use of heterologous vaccine candidates

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Rotavirus is an important cause of morbidity and mortality worldwide and vaccines are currently under development, with clinical trials conducted in humans worldwide. The immune responses in infant BALB/c mice were examined following oral inoculation with murine rotavirus EDIM (2 × 10⁴ focus-forming units) and with three CsCl gradient-purified fractions of heterologous simian rotavirus SA11 (standardized at 2 × 10⁶ CCID⁵₀) that differed in antigen composition: fraction 1 was enriched for double-layered rotavirus particles, fraction 2 for triple-layered particles and fraction 3 consisted mainly of cell components. Diarrhoea and high IgG responses, but marginal IgA responses, were observed after inoculation with all three SA11 fractions. Virus shedding was observed in all EDIM-inoculated mice, but in none of the SA11-inoculated mice. Rotavirus-specific IgG1 : 2a ratios were similar in mice inoculated with EDIM and SA11 fraction 1, but higher for SA11 fraction 3- and lower for SA11 fraction 2-inoculated mice. A higher IgG1 : 2a ratio indicates a more Th2-like immune response. This undesirable response is apparently mostly induced by inoculation with heterologous rotavirus in the presence of abundant cell-associated and soluble rotavirus proteins, compared with infection with a more purified preparation or with homologous virus. These data show that, following inoculation with a standardized amount of infectious virus, the composition of the fraction influences the outcome of the immune responses significantly.

INTRODUCTION

Immune-mediated gastrointestinal disorders, such as classic IgE-mediated food allergy (Moon & Kleinman, 1995), eosinophilic gastroenteritis (Torrieri et al., 1988), allergic colitis (Sherman & Cox, 1982) and eosinophilic oesophagitis (Rothenberg et al., 2001), are occurring with increased frequency in developed countries. In all of these primarily polygenic allergic disorders, Th2-type immune responses are involved. Apparently, in an increasing number of infants, the immune system is skewed towards Th2.

In the so-called 'hygiene hypothesis' (Strachan, 1989), it has been postulated that reduced exposure of young children to common infections of childhood may lead to delayed maturation of the immune system, reflected by skewing of the Th1:Th2 balance in favour of Th2. This skewing towards a Th2-type immune response results in an increased likelihood of developing IgE-mediated allergies in atopic individuals. Matricardi et al. (2000) suggested a role of enteric pathogens in the maturation/skewing of the immune system, following the observation that allergic diseases in 20-year-old military recruits who had a history of exposure to food and orofaecal pathogens were reduced by 60% compared with recruits without signs of exposure (Matricardi et al., 2000, 2002).

Rotavirus is a common enteric pathogen causing infection in early childhood. Even in developed countries, the great majority of children under 5 years of age experience at least one rotavirus infection (de Wit et al., 2000). Worldwide, approximately 440 000 children die each year from rotavirus diarrhoea, mostly in developing countries (Parashar et al., 2003). For that reason, rotaviruses have received a high priority as a target for vaccine development. Most current
rotavirus-vaccine strategies are based mainly on the use of heterologous (animal) rotaviruses to immunize humans against subsequent infections with homologous (human) rotavirus [reviewed by Ward (2003)].

Studies of natural rotavirus infection have relied, for a major part, on the mouse model. Infections with murine rotavirus result in diarrhoea through epithelial dysfunction and the effects of the virus-encoded enterotoxin NSP4 when given before 15 days of age, after which mice no longer experience clinical signs, but shed the rotavirus in stools for 7 days after oral inoculation (Ward et al., 1990; Burns et al., 1995; Rose et al., 1998). Non-murine (heterologous) rotaviruses can be used in the same experimental design as a model for rotavirus vaccine, thus allowing us to compare wild-type (homologous) infection with vaccination (Feng et al., 1994; Fromantin et al., 1998). For these infections, however, studies on virulence and replication in the gastrointestinal epithelium in vivo are limited (Gouvea et al., 1986; Bell et al., 1987; Majerowicz et al., 1994). The dose of heterologous simian strains required to induce diarrhoea in suckling mice is about $10^5$–$10^6$ times higher than that required for a homologous murine virus strain (Feng et al., 1994). For that reason, most studies on oral heterologous rotavirus infections are done with unpurified tissue-culture concentrates of rotaviruses. These crude virus preparations contain a mixture of complete and incomplete virus particles, as well as cellular proteins, and are therefore very different in antigenic composition from the (low-dose) inoculum used in a natural rotavirus infection. This lack of standardization makes a direct comparison of the data on rotavirus pathogenesis and immune responses for the different viruses very difficult. As part of ongoing studies on the hygiene hypothesis, in which we measure the effects of rotavirus exposure on immune maturation and allergic challenge in young mice, we analysed differences in the immune responses stimulated by different purified simian rotavirus SA11 fractions. Furthermore, we examined the pathogenesis of a heterologous rotavirus infection by examining the induction of diarrhoea and timing and extent of rotavirus replication in the gastrointestinal epithelium in mice.

**METHODS**

**Virus and antigen/inoculum preparation.** Two viruses were used in this study: the simian rotavirus SA11 was obtained from the ATCC and the unpassaged murine EDIM (Epizootic Diarrhoea of Infant Mice) strain was provided by Dr R. Ward, Children's Hospital Research Foundation, Cincinnati, OH, USA. The EDIM strain was titrated by fluorescent focus assay in monkey kidney MA104 cells (Ward et al., 1990). SA11 rotavirus was plaque-purified serially three times by limiting dilution in MA104 cells. SA11 virus (activated by adding $10 \mu g$ trypsin ml$^{-1}$ at $37^\circ C$ for 1 h) was subsequently grown at an m.o.i. of 0.1 on MA104 cells attached to Cytodex-3 microcarriers (Amersham Biosciences) in a 3 l spin-culture flask (Wheaton Science Products) in Eagle's minimal essential medium supplemented with 2 mM glutamine, 100 $\mu g$ penicillin ml$^{-1}$, 100 $\mu g$ streptomycin ml$^{-1}$, 0.125% NaHCO$_3$ and 0.5 $\mu g$ trypsin ml$^{-1}$ (Sigma Chemical Company). Cells and medium were harvested when 100 % cytopathological effect (CPE) was observed and subjected to one cycle of freezing and thawing. The lysate was extracted with 10% arklone (trichlorotrifluoroethane; ICI), centrifuged for 30 min at 3000 r.p.m. and the aqueous phase was concentrated by ultrafiltration through a 10 000 MW membrane (Millipore). Finally, CsCl was added to the suspension to reach a final density of 1.37 g ml$^{-1}$. Rotavirus particles and cellular components were separated by density-gradient centrifugation [35 000 r.p.m., 18 h, 4°C in a SW 40Ti rotor (Beckman)]. Three fractions enriched for triple-layered (tl; 1.34 g cm$^{-3}$) and double-layered (dl; 1.36 g cm$^{-3}$) rotavirus particles, and cellular/virus proteins (1.26 g cm$^{-3}$) were collected and dialysed extensively against PBS. For each preparation, the infectivity and (predominant) type of particles were determined by virus-titration assays on MA104 cells and electron microscopy (EM). The SA11 inocula were standardized to contain 2 x $10^6$ CCID$_{50}$ (cell-culture infectious dose 50%) per 100 $\mu$l PBS. The EDIM inoculum was prepared to contain 2 x $10^6$ focus-forming units (f.f.u.) per 100 $\mu$l PBS.

Purified SA11 rotavirus ELISA antigen was obtained by centrifugation of the concentrated virus suspension (ultrafiltrated as described above) through a 40 % sucrose cushion. The pellet was resuspended in PBS by vigorous pipetting and stored frozen at $-20 ^\circ C$ in aliquots. Untreated virus suspensions were exposed to UV-B radiation in 24 wells (area, 1 cm$^2$) on melting ice (to prevent heating effects and evaporation) for 45 min. The UV source (STX-35 M; UVitec) emitted UV-B (280–320 nm) at 0.43 mJ cm$^{-2} s^{-1}$, measured at the sample level with an Optronic OL-752-PMT spectroradiometer. Samples were titrated immediately after exposure.

**Preparation of monoclonal antibody (mAb) AC3H7.** This antibody was used for many years in our laboratory and produced as follows. BALB/c mice (6–10 weeks old) were immunized subcutaneously with 30 $\mu g$ CsCl-purified tl SA11 rotavirus emulsified in complete Freund’s adjuvant, a second immunization 2 weeks later with 60 $\mu g$ and a final intravenous injection 1 week later with 100 $\mu g$ purified tl SA11 rotavirus. Fusion of a single-cell suspension of splenocytes with myeloma P3-X63-Ag8653 cells was performed 3 days after the last vaccination, using 40 % polyethylene glycol and selection in HAT medium (Westerwoudt et al., 1984).

Supernatants of wells with cell growth were screened for rotavirus-specific IgG antibodies in an enzyme immunoassay as described below. Antibody-secreting hybridomas were cloned twice by limiting dilution. Five million viable cells were injected intraperitoneally into BALB/c mice previously primed with pristane (Sigma-Aldrich). Ascitic fluid was harvested 1–2 weeks after cell inoculation and stored at $-70 ^\circ C$.

**Animals.** Pregnant BALB/c dams seronegative for rotavirus antibodies as determined by ELISA were obtained from Harlan CPB at least 1 week before partus and housed in microisolator cages containing sterile bedding, water and ovalbumin-depleted food. All pups used in the experiments were between 6 and 8 days of age. Experiments were reviewed and approved by the Ethics Committee of the National Institute for Public Health and the Environment of the Netherlands.

**Virus inoculation.** Litters of 6–8-day-old BALB/c mice were infected orally with 100 $\mu$l inoculum per mouse. Control animals were inoculated with concentrated mock-infected cell lysate prepared as for the virus concentrate. Infected pups were examined daily for diarrhoea by gentle palpation of the abdomen. Stools were scored from 0 to 4 based on colour, texture and amount of stool. Normal faeces were scored as 1, loose faeces as 2, yellow faeces as 3 and watery faeces as 4. Stools with a score of $\geq 2$ were considered as diarrhoea (Ball et al., 1990). The daily proportion of diarrhoeal samples was calculated by dividing their total number by the total number of samples collected each day.

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Mice were anaesthetized intraperitoneally with barbiturate/chloral hydrate and sacrificed. Segments of duodenum, jejunum and ileum were collected immediately after euthanasia at different time points (8 h and 1, 2, 4, 7, 10 and 14 days) post-infection for five infected and three control mice per time point. Gut sections were flushed in cold PBS, fixed in 4% (w/v) paraformaldehyde (Merck) for 4 h and embedded in paraffin wax by using routine procedures (Verburg et al., 2000). Twenty-one days after oral infection, blood samples were taken by retro-orbital puncture. Sera were stored at 22°C before testing.

Immunohistochemistry and in situ hybridization. To determine the presence of replicating virus, immunohistochemistry and in situ hybridization were used. Paraffin-embedded tissues were sectioned (5 μm), deparaffinized with xylene (Merck) and rehydrated in graded ethanol solutions. Sections were incubated overnight at 4°C using rabbit polyclonal antibodies K3PPIV (1/1000) directed against rotavirus SA11 and rabbit polyclonal antibodies directed against the rotavirus non-structural protein NSP4 (1/1000) as described previously (Boshuizen et al., 2003; Renes et al., 2002).

In situ hybridization was performed as described previously, using non-radioactive digoxigenin (DIG)-11-UTP-labelled 750 bp NSP4 (SA11 strain) hydrolysed in 80 mM NaHCO3 and 120 mM Na2CO3 (pH 10.2) to obtain probes of various lengths (Cox et al., 1984; Boshuizen et al., 2003).

Detection of serum antibodies. IgG, IgG1, IgG2a and IgA levels were determined by ELISA. After each incubation step, microtitre plates were washed four times with wash buffer (PBS supplemented with 0.05% Tween 20). Optimal dilutions of antigen, serum and conjugate were determined by checkerboard titration.

Total IgG1 and IgG2a antibody levels were measured by coating 96-well maxisorp microtitre plates (Nalgene Nunc International) with a 1/1000 dilution of polyclonal goat anti-IgG1 or -IgG2a serum (Sigma Chemical Company) in coating buffer [0.05 M sodium carbonate buffer (pH 9.6)]. Serum samples were diluted 1/5000 in dilution buffer (PBS plus 0.5% Tween 20 plus 1% BSA) and loaded onto the plates. After 1 h incubation at 37°C, the plates were incubated with phosphatase-conjugated goat anti-mouse IgG (Sigma Chemical Company) for 1 h at 37°C and detected with the substrate p-nitrophenylphosphate at a concentration of 1 mg ml−1 in 0.1 M glycine buffer (pH 10.4) at room temperature for 10 min. A405 values were measured in an ELISA reader (Organan Teknika).

Rotavirus-specific IgG levels were measured by coating 96-well maxisorp microtitre plates (Nalgene Nunc International) with a 1/100 dilution of polyclonal rabbit anti-SA11 serum (K3PPIV) in coating buffer. After an overnight incubation at 4°C, 100 μl sucrose-purified SA11 virus diluted 1/100 in dilution buffer was added and incubated for 1 h at 37°C.

Subsequently, plates were loaded with 1/50-diluted mouse serum samples in dilution buffer and a pool of known positive and negative sera from rotavirus-infected and mock-infected mice, respectively, as controls. Plates were incubated for 1 h at 37°C. The detector, phosphatase-conjugated goat anti-mouse IgG (Sigma Chemical Company) or polyclonal goat anti-IgG1 or -IgG2a serum (Sigma Chemical Company), was added for 1 h at 37°C. Incubation of the substrate p-nitrophenylphosphate at a concentration of 1 mg ml−1 in 0.1 M glycine buffer (pH 10.4) was at room temperature for 10 min. A405 values were measured in an ELISA reader (Organan Teknika). A sample was considered positive when the A405 was above the cut-off levels (mean ± 3SD of the samples of non-immunized mice).

For the detection of rotavirus-specific IgA titres in serum, a capture ELISA was performed. Briefly, 96-well microtitre plates (Nalgene Nunc International) were coated overnight at 4°C with goat anti-mouse IgA (Sigma Chemical Company) in coating buffer. Serum samples were diluted 1/100 in dilution buffer and added to the plates. After 1 h incubation at 37°C, the plates were incubated with sucrose-purified SA11 virus diluted 1/100 in dilution buffer for a further 1 h at 37°C. Anti-rotavirus SA11 mAb (AChH7) conjugated to horseradish peroxidase was added for 1 h at 37°C as a detector. After incubation with the substrate 3,3’,5,5’-tetramethylbenzidine (Sigma Chemical Company) for 5 min at room temperature, A450 values were measured in an ELISA reader (Organan Teknika). The test was considered valid when the A450 of the known positive-control serum was above 1000 and a sample was considered positive when the A450 was above the cut-off levels (mean ± 3SD of negative sera from mock-infected mice).

Detection of virus shedding. Intestinal contents were collected from each sacrificed mouse at different time points (0–14 days) post-infection and stored at −20°C. Faecal suspensions (10%) were prepared in PBS, clarified by centrifugation (1000 g for 5 min) and analysed by ELISA using a Premier Rotacloane kit (Meridian Diagnostics) according to the manufacturer’s instructions, or cultured on MA104 cells.

Statistical analysis. Statistical analysis of all data was performed by using Student’s t-test for unpaired data (two-tailed). Data were expressed as the mean ± SEM and P values of ≤0.05 were considered statistically significant.

RESULTS

Virus preparation

CsCl fractionation of SA11-infected cell supernatant yielded three distinct visible bands in the gradient, containing dl and tl particles and cell components (mix), respectively, as determined by EM (Fig. 1). Virus titres were 106.1, 1010.0 and 107.6 ml−1, respectively. The final rotavirus-antigen concentrations of the inoculated fractions as measured by ELISA were higher in fractions 1 (dl) (A450 value 900) and 3 (mix) (A450 value >1400) than in fraction 2 (tl) (A450 value 200), as shown in Fig. 1. The protein concentrations of the inoculated SA11 fractions and mock were: fraction 1 (dl), 8 μg ml−1; fraction 2 (tl), 1 μg ml−1; fraction 3 (mix), 1.5 mg ml−1; concentrated mock-infected cell lysate, 1 mg ml−1.

Diarrhoea and virus shedding in newborn mice inoculated with different fractions of simian SA11 and murine EDIM rotavirus strains

The development and duration of diarrhoea after inoculation with the different fractions of the heterologous SA11 rotavirus (2 × 106 CCID50) and homologous EDIM (2 × 104 f.f.u.) over a period of 7 days varied among the different CsCl fractions compared with wild-type virus strain (Fig. 2). All virus preparations induced diarrhoea in the newborn animals. In mice inoculated with the EDIM strain and SA11 fraction 3 (enriched with cell components), diarrhoea was observed from day 1 after inoculation in 45 and 30% of the animals, respectively, whereas diarrhoea was observed from day 2 onwards in the groups inoculated with the other gradient-purified fractions of SA11. No diarrhoea was observed in the mice inoculated with UV-inactivated SA11 fraction 2 (enriched for tl rotavirus particles). Virus shedding in the EDIM-inoculated group was observed from...
day 2 until day 10, as reported previously (Boshuizen et al., 2003), and no virus shedding was found in the SA11-infected animals (data not shown).

Serum antibody responses in mice inoculated with different preparations of SA11 and the EDIM rotavirus strain

Systemic IgG1 and IgG2a responses and rotavirus-specific antibody responses (IgA, IgG, IgG1 and IgG2a) were measured in sera of individual mice at day 21 after inoculation. High rotavirus-specific IgG responses were obtained in the EDIM-infected group and in mice inoculated with SA11 fractions 1, 2 and 3 (Fig. 3a). The mean IgG responses were significantly higher in the SA11 fraction 3-infected animals compared with EDIM or the other fractions \((P<0.05)\). The IgA responses, on the other hand, were much lower in all SA11-infected animals than in the EDIM-infected animals (Fig. 3b).

To examine the role of replication in the induction of IgA and IgG responses, we inoculated mice orally with UV-inactivated SA11 tl virus particles (fraction 2) corresponding to an original titre of \(2 \times 10^6\) CCID\(_{50}\). We found a marginal IgG (one positive out of 25 mice) and no IgA response in these mice (Fig. 3), similar to in the mock-infected animals.

We then tested sera for specific IgG subtype responses, plotted as the IgG1 : IgG2a ratios of SA11 compared with the EDIM group (Fig. 4). The IgG1 : IgG2a ratios of specific antibodies in sera from mice inoculated with EDIM and fraction 1 (dl) of SA11 were similar, but a significantly higher ratio was observed in the mice infected with fraction 3 of SA11. Furthermore, the fraction 2 (tl) serum IgG1 : IgG2a ratio of SA11-infected mice was significantly lower than that of EDIM-infected mice and fraction 3 (Fig. 4).

Histological changes in the small intestine during rotavirus infection

Histopathological changes in the small intestine were observed at 1 day post-infection (p.i.) and persisted through 7 days p.i. In the EDIM-infected as in the SA11 rotavirus fraction 2-infected animals, throughout the whole intestine (duodenum, jejunum and ileum), these changes were characterized by swollen villus tips with enterocytes containing large vacuoles, shown for 2 days p.i. in the jejunum (Fig. 5). After 7 days p.i., almost-complete resolution of the histopathological changes was observed (data not shown). In control animals, no vacuoles were seen at any time point. To determine the presence of replicating rotavirus in the small intestine, NSP4 and rotavirus structural proteins were detected by immunohistochemistry and NSP4 mRNA was detected by \textit{in situ} hybridization. At 2 days p.i., rotavirus structural protein, NSP4 protein and mRNA were detected in almost all epithelial cells of the upper part of the small-intestinal villi of EDIM-infected mice. No rotavirus structural proteins or mRNA was
detected in any section of the small intestine of SA11-infected or control mice at any time point (Fig. 5). However, in SA11-infected mice, anti-NSP4 (114–135) rabbit polyclonal serum stained crypt cells of the small intestine (jejunum) (Fig. 5). No staining was observed with the NSP4 antiserum in the intestine of control mice after mock infection (Fig. 5).

**DISCUSSION**

In most studies of immune response against heterologous rotavirus infections, crude concentrates of cell culture-grown virus are used (Merchant *et al.*, 1991; Feng *et al.*, 1994; McNeal *et al.*, 1994; Ishida *et al.*, 1997; Fromantin *et al.*, 1998; Katyal *et al.*, 1999). That means that, in addition to the virus, these preparations contain unknown and non-standardized amounts of cellular proteins. We postulated that the type of purification of the viruses used in such infection experiments might influence the ensuing immune responses, thus leading to potentially ambiguous conclusions. The present study was designed to gain more insight into the influence of different preparations of the simian SA11 rotavirus on the immune response following oral delivery to suckling BALB/c mice. We found that each of the purified fractions of SA11, corresponding to the two forms of virus particles (tl and dl) and infected-cell components, is able to induce diarrhoea in 7-day-old suckling BALB/c mice, but with different kinetics. Even though the fractions are CsCl gradient-purified, they do not contain the specified components exclusively; infectious virus was present in all fractions administered, but in different amounts. The three
SA11 preparations were standardized to $2 \times 10^6 \text{CCID}_{50}$ per mouse and the effects of the different fractions were compared. Mice infected with EDIM were used as controls to confirm the response to a homologous rotavirus infection and to assess the effect of a homologous infection on immune maturation in our model. Standardization of the SA11 fractions resulted in great differences in antigen content of the preparations. Nevertheless, similar serum IgG antibody responses were found in the different groups, with a slightly higher response in the mice infected with the fraction with the highest concentration of cell components, which had the highest antigen content (fraction 3). The IgG subtype responses were not distributed equally and we found a significantly higher rotavirus-specific IgG1 : 2a ratio in the animals inoculated with fraction 3, compared with the other fraction- and the EDIM-infected animals. This indicates a more Th2-like immune response following infection with rotavirus in the presence of abundant cell-associated and rotavirus proteins, compared with infection with a more purified preparation or with EDIM (Fireman, 2003). This shows that the composition of the fraction influences the outcome of the immune response following infection with the same amount of infectious virus. This ‘adjuvant’ effect may be the result of the increased amount of antigen/protein to which these mice are exposed or may result from adjuvant effect of an unknown component of infected cells. The effect of different preparations of orally administered (vaccine) virus has become increasingly relevant following the finding of an as-yet-unexplained adverse effect (i.e. intussusception) of oral rotavirus vaccine (Murphy et al., 2001). This raised questions about the exact effect of heterologous rotavirus infection on the (neonatal) gut. There are additional reasons for this increased interest (see below).

Fig. 5. Histological analysis of rotavirus replication in the small intestine in neonatal mice 2 days post-oral inoculation with mock control (a, d, g), CsCl-purified fraction 2 of simian SA11 strain (b, e, h, j) or rotavirus murine EDIM strain (c, f, i). Sections were stained by using polyclonal anti-rotavirus antigen (a–c), NSP4 protein expression in jejunum was detected by immunohistochemistry (d–f, j) and NSP4 mRNA was detected by in situ hybridization (g–i).
Immune-mediated gastrointestinal disorders, including classic IgE-mediated food allergy (Moon & Kleinman, 1995), eosinophilic gastroenteritis (Torpier et al., 1988), allergic colitis (Sherman & Cox, 1982) and eosinophilic oesophagitis (Rothenberg et al., 2001), are primarily polygenic allergic disorders that involve Th2-type immune responses and are occurring with increased frequency in developed countries. Concomitantly, an increased prevalence of Th1-driven diseases, such as inflammatory bowel disease (Walsh & Gaginella, 1991; Targan & Murphy, 1995), is seen. Therefore, a better understanding of the factors that influence the development of a balanced mucosal immune response is essential. Mucosal vaccinations are part of the challenge of the immunological balance.

IgA titres were lower for the three fractions of SA11 than for the EDIM strain, despite comparable IgG titres. This observation has been found previously (Shaw & Hempson, 1996) and may be related to reduced replication in the intestine of the heterologous SA11 strain compared with the murine EDIM strain, as replication is an important determinant of IgA responses to rotavirus (Feng et al., 1994). Indeed, we did not find measurable virus shedding in the SA11-infected animals, whereas virus antigen was detected readily in faeces of the EDIM-infected animals. Contradictory to this observation is the fact that we did find diarrhoea in infected animals up to 1 week after oral delivery of the SA11 virus. This suggests that replication took place, as NSP4 or its cleavage product NSP4 (112–175), thought to be important for the induction of diarrhoea in young siblings, is not found in mature infectious virus particles and is only synthesized within the host cells during virus replication (Ball et al., 1996; Zhang et al., 2000). We cannot exclude the possibility of contamination with a small amount of NSP4 protein in the t and dl particle fractions produced during virus growth in cell culture.

Ball et al. (1996) reported that diarrhoea was observed within 1–4 h of inoculation of purified NSP4, which continued for up to 8 h, but occasionally persisted for 24 h. Similarly, Shaw and coworkers suggested that rotavirus replication is not a fundamental requirement for the induction of disease, but a virus-toxin-like effect (Shaw et al., 1995). To study the effects of heterologous infection further, we examined intestinal sections from the mice infected with SA11 fraction 2 or EDIM. We observed large vacuoles in the small-intestinal enterocytes of the villus following exposure to rotavirus SA11, similar to those observed after EDIM infection (Boshuizen et al., 2003). As indicated by the lack of virus shedding, we could not detect virus replication in the small intestine by either immunohistochemistry or in situ hybridization in SA11-infected mice, in contrast to observations in the EDIM-inoculated mice. This suggests that replication may have occurred extraintestinally, as shown by Uhnoo et al. (1990), who found that infection with a heterologous rhesus rotavirus resulted in hepatitis in 84% of SCID and 21% of BALB/c mice. Furthermore, Blutt and coworkers showed viraemia and antigenaemia in infected patients and in animal models, suggesting that extraintestinal spread might be a common event (Blutt et al., 2003). Recently, two reports have confirmed the extraintestinal replication of homologous and heterologous rotaviruses in different organs of neonatal BALB/c mice and rats (Crawford et al., 2006; Fenaux et al., 2006). We cannot rule out intestinal replication, as only a small segment of the different regions was analysed by immunohistochemistry and in situ hybridization. However, replication of the heterologous SA11 virus at a non-mucosal site could explain the low induction of (mucosal) IgA with a high systemic IgG response.

In case of extraintestinal replication of the virus, histological damage and diarrhoea might be induced by a secreted virus factor and not by direct virus CPE (Osborne et al., 1988; Ciarlet et al., 2002). A candidate factor could be NSP4 or its cleavage product NSP4 (112–175), which is thought to mediate cell signalling by increasing intracellular calcium levels, leading to chloride secretion in uninfected cells and diarrhoea in young mice (Ball et al., 1996; Zhang et al., 2000). This secretory mechanism, however, is thought to act in the intestinal crypts (Lundgren & Svensson, 2001). We found staining with the anti-NSP4 antibody in the crypt cells of the small intestine of SA11-infected/inoculated animals with diarrhoea and without measurable replication. It is possible that low amounts of NSP4 are present in the SA11 fraction and that these low levels of NSP4 are able to induce diarrhoea. However, the possibility of an incomplete replication cycle of SA11 in the mouse intestine, as shown by Kitamoto and coworkers in HEPG2 cells, combined with a very early secretion (25 h p.i.) of the functional cleavage product of NSP4 enterotoxin peptide (aa 112–175) (Kitamoto et al., 1991; Zhang et al., 2000) could not be ruled out completely.

In concurrence with the hypothesis stated above, we have shown that, after EDIM infection of mice, NSP4 is produced in and secreted basolaterally from infected enterocytes (Boshuizen et al., 2004). It is tempting to speculate that the basolaterally secreted NSP4 can subsequently be transported via the bloodstream to the small intestine and initiate pathological damage and diarrhoea in inoculated mice.

In conclusion, we show that the polarization of the immune response (Th1:Th2 balance) as a result of heterologous rotavirus inoculation is dependent on the exact composition and purification of the inoculum used. Furthermore, it is demonstrated that the mucosal immune response is lacking after a heterologous infection by SA11. These factors should be evaluated with new rotavirus vaccines in upcoming/ongoing trials, as they may have a significant impact on the neonatal immune system.

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