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Homeostasis and function of goblet cells during rotavirus infection in mice

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Abstract

Rotaviruses are the leading cause of severe viral gastroenteritis in young children. To gain insight in goblet cell homeostasis and intestinal mucin expression during rotavirus infection, 6-day-old mice were inoculated with murine rotavirus. To determine epithelial cell migration, mice were injected with BrdU just before inoculation. Small intestines were isolated at different days postinfection (dpi) and evaluated for rotavirus and goblet cell-specific gene expression. Small intestinal mucins of control and infected animals at 1, 2, and 4 dpi were isolated and tested for their capability to neutralize rotavirus infection in vitro. After inoculation, two peaks of viral replication were observed at 1 and 4 dpi. During infection, the number of goblet cells in infected mice was decreased in duodenum and jejunum, but was unaffected in the ileum. Goblet cells in infected animals accumulated at the tips of the villi. Muc2 mRNA levels were increased during the peak of viral replication at 1 dpi, whereas at other time points Muc2 and Tff3 mRNA levels were maintained at control levels. Muc2 protein levels in the tissue were also maintained, however Tff3 protein levels were strongly decreased. The number of goblet cells containing sulfated mucins was reduced during the two peaks of infection. Mucins isolated at 1 and 2 dpi from control and infected mice efficiently neutralized rotavirus infection in vitro. Moreover, mucins isolated from infected mice at 4 dpi were more potent in inhibiting rotavirus infection than mucins from control mice at 4 dpi. In conclusion, these data show that during rotavirus infection, goblet cells, in contrast to enterocytes, are relatively spared from apoptosis especially in the ileum. Goblet cell-specific Muc2 expression is increased and mucin structure is modified in the course of infection. This suggests that goblet cells and mucins play a role in the active defense against rotavirus infection and that age-dependent differences in mucin quantities, composition, and/or structure alter the anti-viral capabilities of small intestinal mucins.
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Keywords: Intestinal homeostasis; Goblet cells; Rotavirus infection

Introduction

Rotavirus is the major infectious agent in the development of gastroenteritis, malnutrition, and diarrhea in young children and animals (Estes, 2001; Kapikian et al., 2001). In developed countries, mortality rates are low and illness is usually self-limiting (Uhnoo et al., 1988). In developing countries, however, each year about 440,000 children of under 5 years of age die due to infection with rotavirus (Parashar et al., 2003). Mortality is essentially related to dehydrating diarrhea. So far, no vaccine is available. Therefore, it is important to develop alternative therapies based on a better knowledge of rotavirus pathogenesis.

Rotavirus binding and entry of host cells is thought to be a multifactorial process (Lopez and Arias, 2004). Sialic acid-dependent rotavirus strains use sialic acid (SA) residues on the surface of target cells for initial attachment

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(Delorme et al., 2001; Rolsma et al., 1998; Superti and Donelli, 1991). The initial cell surface receptors containing sialic acid may be either glycolipids or glycoproteins (Delorme et al., 2001; Fiore et al., 1991; Rolsma et al., 1998; Superti and Donelli, 1991; Yolken et al., 1987). For SA-dependent strains, these receptors are thought to play a key role in cellular entry, since SA-independent strains can infect cells through either the apical or basolateral membrane, while SA-dependent strains infect cells only through the apical membrane (Ciarlet et al., 2001). Most animal and human rotaviruses, nevertheless, are SA-independent (Ciarlet and Estes, 1999). It is, however, important to realize that apparently most rotavirus strains, SA-dependent as well as SA-independent, indeed bind to SA, but rather have different requirements regarding sialic acids on the host cell (Beisner et al., 1998; Jolly et al., 2000). For glycolipids, it was found that the position of SA residues influenced the binding capacity of the different rotavirus strains. SA-dependent rotavirus strains bind to terminal SA residues in glycolipids, while SA-independent rotavirus strains bind glycolipids with internal sialic acids (which are resistant to neuraminidase treatment) (Delorme et al., 2001). The fact that many glycoproteins can inhibit rotavirus infection in vivo and in vitro is possibly based on the ability of rotavirus to bind to SA residues, since treatment with neuraminidase abolishes the inhibitory effect of these agents (Chen et al., 1993; Newburg et al., 1998; Peterson et al., 2001; Willoughby and Yolken, 1990; Yolken et al., 1987). Some studies, however, indicate that removal of sialic acid from glycoproteins does not significantly alter the inhibitory properties of these compounds (Yolken et al., 1994).

Previous studies have shown that rotavirus infection has a profound effect on small intestinal epithelial homeostasis. It was found that rotavirus induced apoptosis, mild villus atrophy, increased epithelial cell turnover, and hampered enterocyte gene expression (Boshuizen et al., 2003; Collins et al., 1988; Katyal et al., 1999; Osborne et al., 1988; Shepherd et al., 1979; Starkey et al., 1986). Rather than concentrating on enterocyte cell function, we wanted to focus our attention on the function of goblet cells during rotavirus infection and the expression of specific gene products that are secreted by these cells. Goblet cells are characteristic for the intestinal epithelium and are known to secrete molecules such as mucins and trefoil factor family peptides that serve protective and healing functions in the gut, respectively (Thim, 1997; Van Klinken et al., 1995). The secretory mucin Muc2, which is the most important structural component of the intestinal mucus layer, is exclusively and abundantly expressed by goblet cells in the small intestine (Tytgat et al., 1994, 1995; Van Klinken et al., 1995, 1999a, 1999b). After synthesis, Muc2 is secreted into the lumen and forms a protective mucus gel layer that acts as a selective barrier to protect the epithelium from mechanical stress, noxious agents, viruses, and other pathogens (Einerhand et al., 2002; Forstner and Forstner, 1994; Strous and Dekker, 1992; Tytgat et al., 1994; Van

Klinken et al., 1995, 1999a, 1999b). Muc2 and other mucins contain many O-linked SA moieties and are important inhibitors of rotavirus infection (Chen et al., 1993; Newburg et al., 1998; Peterson et al., 2001; van Klinken et al., 1999a, 1999b; Willoughby and Yolken, 1990; Yolken et al., 1987, 1994). It was suggested that intestinal mucins may represent a barrier to certain rotaviruses by neutralizing rotavirus infection through direct interaction with the virus, while hindering virus-to-cell attachment (Chen et al., 1993; Yolken et al., 1987). Interestingly, it was found that mucins isolated from suckling mice neutralize rotavirus more effectively than adult mucins (Chen et al., 1993). Besides Muc2, the trefoil factor family peptide 3 (Tff3) is another secretory protein synthesized by goblet cells. Tff3 is a small bioactive peptide that is involved in epithelial repair (Mashimo et al., 1996; Thim, 1997). Because Tff3 acts as a motogen, i.e., promotes cell migration without promoting cell division, it stimulates epithelial restitution and thus epithelial repair (Wong et al., 1999). Therefore, Tff3 could also be involved in both protection against rotavirus infection as well as epithelial restitution after rotavirus-induced injury.

In this paper, we examined the timing and extent of rotavirus replication in the murine small intestine and related this to the number of goblet cells and the production of the goblet cell-specific markers Muc2 and Tff3. Moreover, we performed qualitative analyses of the structural changes of mucins through histochemical staining, and we assessed the ability of isolated intestinal mucins to inhibit rotavirus infection in vitro.

Results

Kinetics of rotavirus replication in the mouse small intestine

In the present study, we used 6-day-old suckling mice and inoculated them orally with 2×10^4 ffu of EDIM rotavirus. As a control, mice of the same age were PBS inoculated. As in a previous study (Boshuizen et al., 2003), rotavirus-inoculated mice developed diarrhea over a period of 5 days starting at 1 day postinfection (dpi). Control mice did not develop diarrhea at any time point (data not shown). As observed by immunohistochemical staining of rotavirus particles or detection of VP4 RNA by in situ hybridization, rotavirus replication was confined solely to the upper villus epithelium (Figs. 1A and B). To determine the kinetics of rotavirus replication in the present experiment, replicating virus in small intestinal tissue was determined by detection of VP4 RNA. In agreement with previous studies (Boshuizen et al., 2003; Osborne et al., 1988; Starkey et al., 1986), two peaks of viral replication were observed in both the jejunum and ileum. At 6 h postinfection (hpi), very low levels of VP4 RNA were detected in each segment (Fig. 1C). At 1 dpi, there was a first peak in the amount of VP4 RNA. The
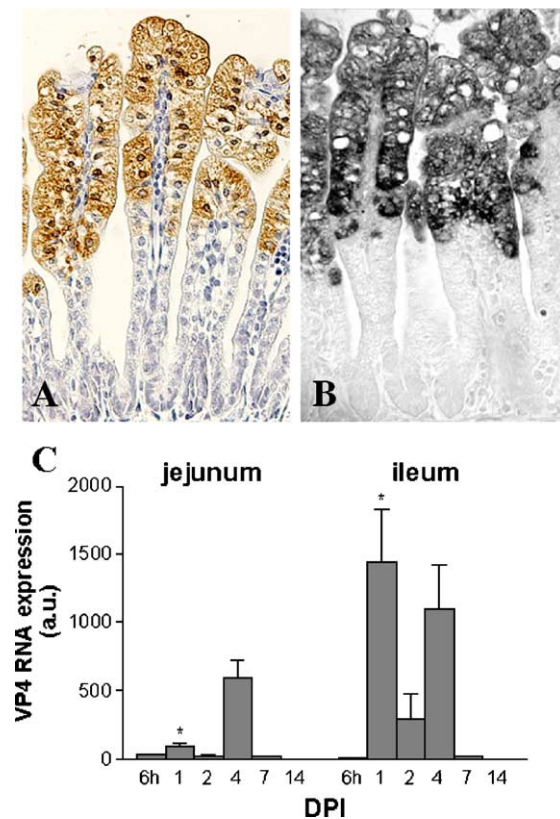
Fig. 1. Rotavirus replication in the mouse small intestine. Rotavirus protein (panel A) and VP4 RNA (panel B) expressions in ileum at 1 dpi were detected by immunohistochemistry and in situ hybridization, respectively. Virus replication was solely confined to the upper villus epithelium. Quantitative expression of VP4 RNA at several days postinfection was analyzed by RNA dot-blotting (panel C). VP4 showed two peaks of expression at 1 and 4 dpi in both jejunum and ileum. At 1 dpi, the level of VP4 RNA was significantly higher in the ileum compared to the jejunum, *$P < 0.05$ (using Student's t test). No VP4 RNA was detected beyond 7 dpi in any part of the small intestinal epithelium. Error bars in panel C represent ± SEM.

VP4 mRNA level was low at 2 dpi, and a second peak of replication was observed at 4 dpi. Thereafter replication decreased to very low (7 dpi) and finally undetectable

levels (14 dpi). As in our previous studies, the extend of virus replication in the ileum was more extensive than in the jejunum.

Number of goblet cells during rotavirus infection

We performed AB-PAS staining on intestinal tissue sections and counted the number of goblet cells per villus in duodenum, jejunum, and ileum. In control mice, the number of goblet cells per villus in the duodenum, jejunum, and ileum remained constant during development in the second week after birth (i.e., 6 hpi to 7 dpi, Fig. 2). We however observed a difference in goblet cell numbers during infection, when the duodenum and jejunum were compared to the ileum. During infection, the number of goblet cells in the duodenum and jejunum was significantly decreased at 1 and 2 dpi and 1, 2, and 4 dpi, respectively. In contrast, in the ileum, numbers of goblet cells in infected animals remained comparable to controls during infection. Thus, in the proximal parts of the small intestine, there seems to be an early loss of goblet cells. In order to detect apoptotic goblet cells in the different regions of the small intestine in control and infected mice, we performed a double staining of alcian blue and the apoptotic marker (cleaved) caspase-3. With this double staining, however, we never observed apoptotic goblet cells (i.e., caspase-3 positive AB staining cells, data not shown) after screening numerous intestinal sections.

Rotavirus infection alters goblet cell survival and migration kinetics

In order to investigate the effects of rotavirus infection on goblet cell survival and migration in the small intestine, mice were injected with BrdU just before inoculation with rotavirus. Goblet cell kinetics were analyzed by combined AB staining of the goblet cells and detection of incorporated BrdU in epithelial cells. BrdU-labeled goblet cells are
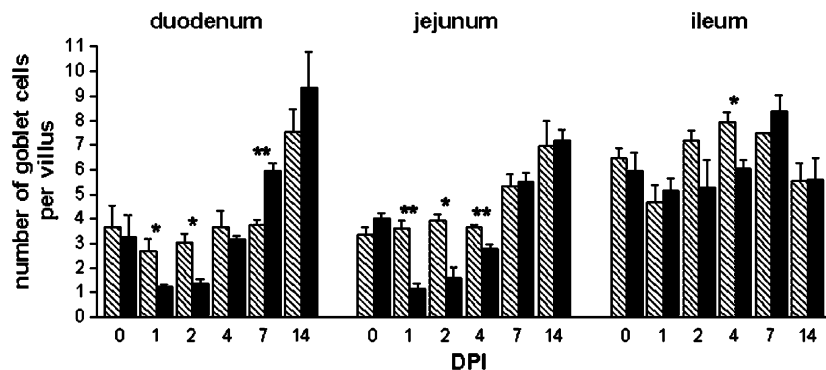

Fig. 2. The numbers of goblet cells per villus in the duodenum, jejunum, and ileum of control mice (hatched bars) and during rotavirus infection (solid bars). During infection, goblet cell numbers in the duodenum and jejunum were significantly decreased at 1 and 2 dpi and 1, 2, and 4 dpi, respectively. In the ileum, goblet cell numbers in infected animals were only significantly decreased at 4 dpi. Data from 3 animals at each time point are expressed as mean number of goblet cells per villus as analyzed after AB-PAS staining ± SEM. Statistical significance in the infected tissue relative to the control tissue is indicated per time point per tissue, *$P < 0.05$, **$P < 0.01$ using Student's t test.

recognized by blue-stained granula and brown-stained nuclei. In control animals, BrdU-labeled enterocytes migrated in cohorts along the villus epithelium (Figs. 3A–D), and at 7 dpi the enterocytes had reached the villus tips. Goblet cells had a different migration pattern and remained evenly distributed in the villus epithelium as can be seen at 7 dpi (Fig. 3D). BrdU-labeled cells in infected mice in general migrated faster up the villus than in control animals. BrdU-labeled enterocytes were already lost from the villi in infected animals at 4 dpi. In infected mice, goblet cells where not evenly distributed along the villi during infection (1, 2, and 4 dpi). Instead, the goblet cells tended to accumulate at the tips of the villi (Figs. 3E–H). This was most prominent in the ileum, but was also observed in the duodenum and jejunum. At 4 dpi, almost all BrdU-labeled cells, that were still present in the villus epithelium, were goblet cells, located at the tips of the villi. BrdU-labeled goblet cells were even found at the tops of the villi in infected animals at 7 dpi, when all BrdU-labeled enterocytes were lost from the epithelium (Fig. 3H).

Muc2 and Tff3 mRNA and protein levels in jejunum and ileum during rotavirus infection

Our previous findings showed that enterocyte gene expression rapidly deteriorated during rotavirus infection (Boshuizen et al., 2003). In contrast, infection did not seem to have large effects on goblet cell gene expression. Muc2 mRNA remained detectable in goblet cells during the course of infection even at time points of extensive damage and vacuolization of enterocytes (Fig. 4). To examine whether goblet cell-specific expression was quantitatively affected during infection, Muc2 and Tff3 mRNA and protein levels in jejunum and ileum were assessed by RNA and protein spot blotting, respectively. Subsequently, the expression levels were corrected for the numbers of goblet cells in the tissue, as shown in Fig. 2, to obtain values for Muc2 and Tff3 expression per goblet cell. In general, there were only minor differences in the levels of Muc2 and Tff3 mRNAs in the jejunum vs. the ileum. Muc2 mRNA levels were decreased in the jejunum of infected animals when compared to controls (Fig. 5A). In ileum, the Muc2 mRNA levels were comparable with controls, with the exception of the Muc2 mRNA levels 1 dpi that were significantly increased. When these Muc2 mRNA values were corrected for the number of goblet cells in the respective tissues, it appeared that the level of Muc2 mRNA per goblet cell was similar in jejunum and ileum (Fig. 5C). In both jejunum and ileum, there was a peak increase in Muc2 mRNA level per goblet cell at 1 dpi. Tff3 mRNA levels in the jejunum as well as in the ileum remained largely unaltered during infection, i.e., the levels of Tff3 mRNA did not differ from the control values
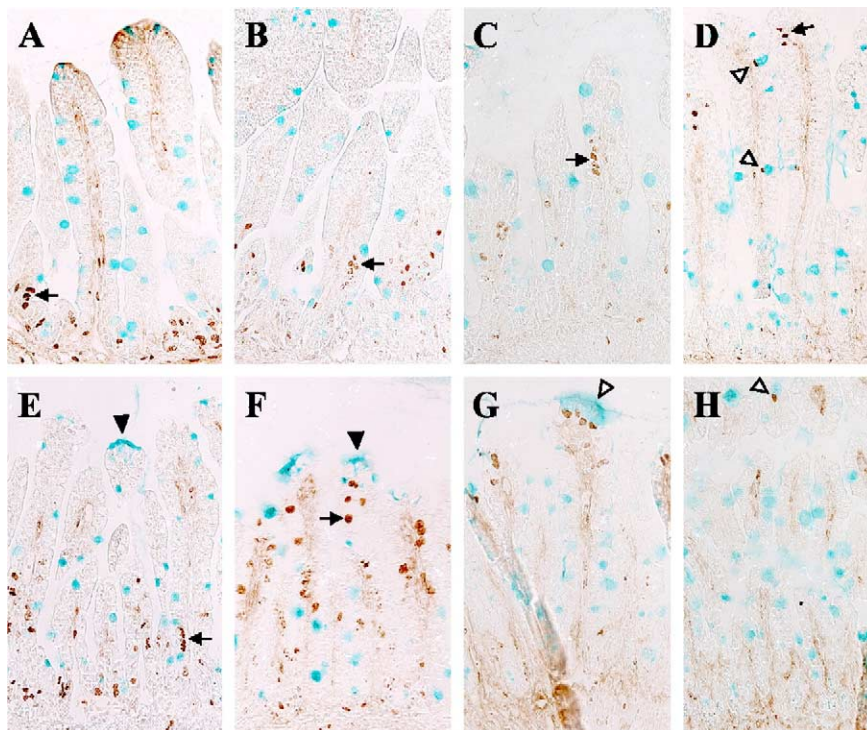

Fig. 3. Goblet cell migration kinetics in the ileum during rotavirus infection. Just before inoculation, mice were injected with BrdU. Goblet cells in control (A–D) and infected mice (E–H) at 1 (A and E), 2 (B and F), 4 (C and G), and 7 dpi (D and H) were detected by AB staining. From 1 to 7 dpi, BrdU-labeled enterocytes (arrows) in infected mice migrated faster up the villus than in control animals. In infected mice, goblet cells (solid arrowheads or open arrowheads when BrdU-labeled) tended to accumulate at the tips of the villi (E–H), whereas in control mice goblet cells appeared evenly distributed in the villus epithelium.
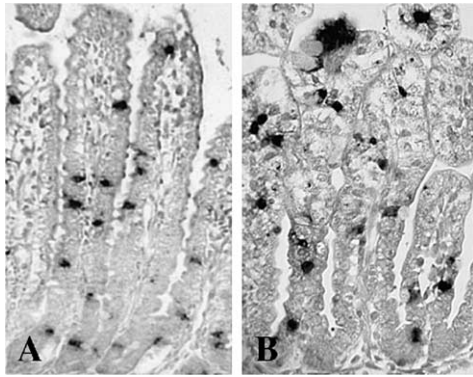
Fig. 4. Muc2 mRNA levels in jejunal sections in control mice (A) and during rotavirus infection (B) as examined by in situ hybridization at 1 dpi. At the time point of massive expression, viral replication and extensive damage and vacuolization of enterocytes, goblet cell-specific Muc2 mRNAs remained detectable and comparable with controls. Original magnification: ×200.

(Fig. 5B). We normalized the Tff3 mRNA levels for the number of goblet cells and found that the Tff3 mRNA levels per goblet cell were not statistically different from control levels (Fig. 5D).

The Muc2 protein levels appeared different in jejunum and ileum. In the jejunum, the Muc2 protein levels were decreased in infected tissue at 1–4 dpi, and after correction for the number of goblet cells the Muc2 protein levels appeared to be significantly increased at 1 and 2 dpi (Figs. 6A and C). In the ileum, the Muc2 protein levels remained largely unaltered during infection both before and after
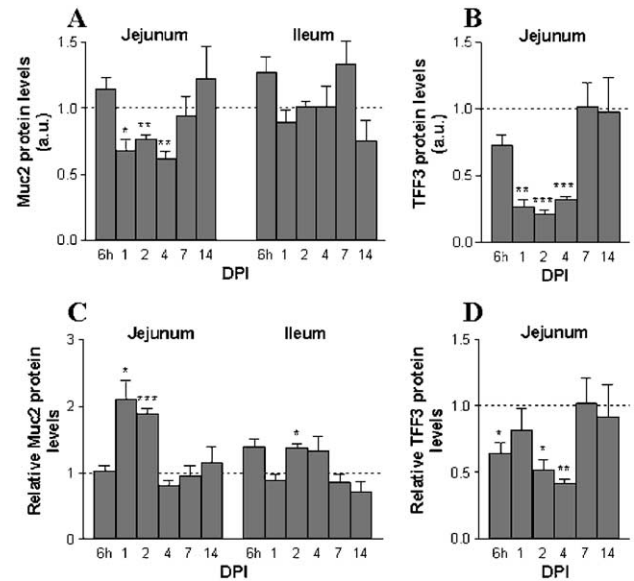

Fig. 6. Quantitative analyses of goblet cell-specific protein levels during rotavirus infection. Muc2 (A) protein levels in jejunum and ileum, and Tff3 (B) protein levels in jejunum during rotavirus infection as determined by protein dot-blot analysis and after normalization by the number of goblet cells in the respective tissue (C and D, respectively). Control values per day were arbitrarily set at a relative expression of 1 and are represented as a dotted line. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control using Student's t test. a.u., arbitrary units. Error bars represent ±SEM.

normalization for the number of goblet cells (Figs. 6A and C). Tff3 protein levels were decreased compared to controls at 6 hpi to 4 dpi (Figs. 6B and D).
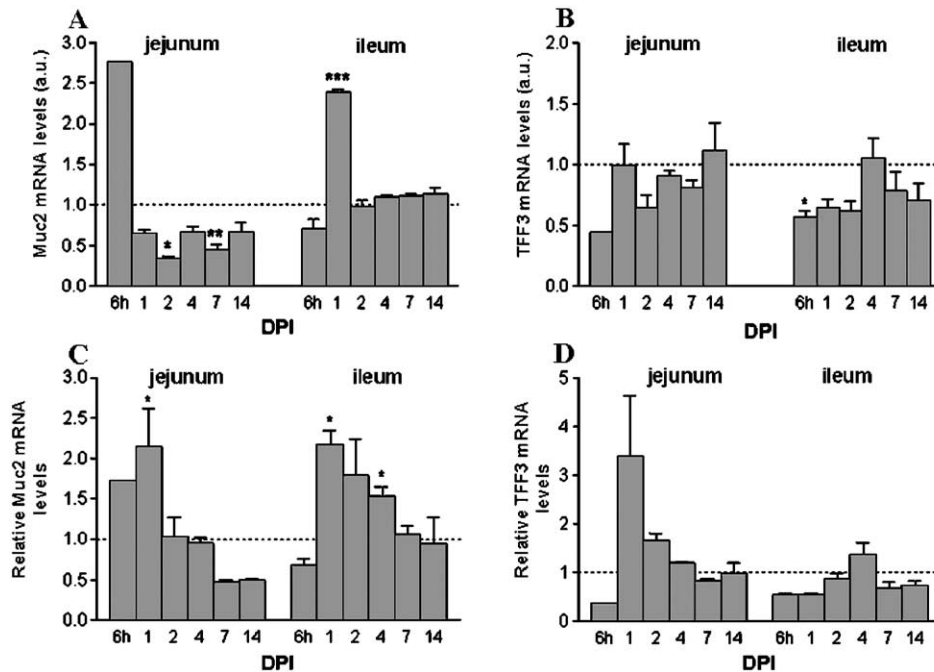

Fig. 5. Quantitative analyses of goblet cell-specific mRNA levels during rotavirus infection. Muc2 (A) and TFff3 (B) mRNA levels in jejunum and ileum during rotavirus infection as measured by RNA dot-blot analysis and after normalization by the number of goblet cells in the respective tissue (C and D, respectively). Control values per day were arbitrarily set at a relative expression of 1 and are represented as a dotted line. The amount of RNA spotted was corrected for GAPDH mRNA expression. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control using Student's t test. a.u., arbitrary units. Error bars represent ±SEM.

Sulfated mucins and sialylated mucins during rotavirus infection

To analyze mucins qualitatively, mucins in control and infected tissues were detected with a combined AB-PAS staining (Fig. 7). With this staining neutral mucins stain magenta, whereas acid mucins stain purple/blue. In the control animals at 4 dpi, goblet cells stained pink, indicating that they primarily contain neutral mucins (Fig. 7A). In infected animals at 4 dpi however, goblet cells stained blue in the crypt region or dark purple on the villi, indicating that these goblet cells contained abundantly acidic mucins (Fig. 7B). The increase in AB-stained cells further suggests that the fraction of acidic mucins increased during rotavirus infection. This phenomenon was observed from 1 to 4 dpi in all analyzed sections from infected animals (Fig. 7B and

data not shown). To further examine mucins qualitatively, the mucins in control and infected ileum were detected with a combined high iron diamine-alcian blue (HID-AB) staining. With the HID-AB staining, sulfated mucins stain black or brown, whereas sialomucins stain blue (Figs. 7C–E). In control mice at 7 dpi, goblet cells predominantly stained black/brown, indicating that they contained sulfated mucins (Fig. 7C). In infected mice at 7 dpi however, many goblet cells stained blue, indicating that they predominantly contained sialomucin (Fig. 7D). We then counted the number of HID-positive goblet cells and represented them as percentage of all goblet cells that were detected by AB-PAS at the villi of control and infected animals (Fig. 7E). During infection, the fraction of HID-stained goblet cells was decreased at 1 and 7 dpi. At 2 dpi, there was a trend towards a decreased number of HID-stained goblet cells compared to controls, but this did not reach statistical significance. These results indicate that at specific time points during rotavirus infection, mucins become less sulfated.
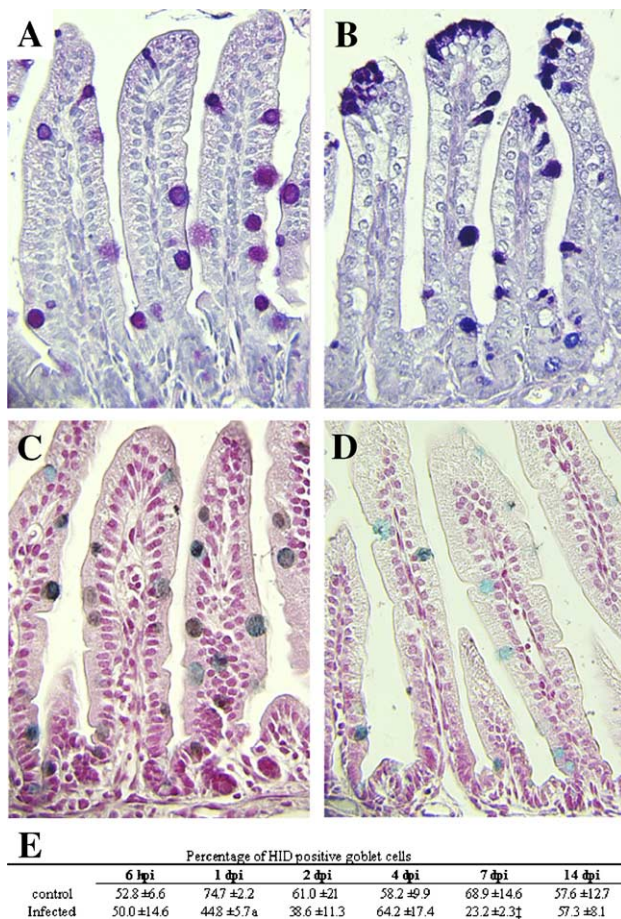

Fig. 7. The number of goblet cells containing sulfated mucins and acidic mucins during rotavirus infection. Using a combined AB-PAS staining, goblet cells in control tissue (A; at 4 dpi) stained pink, whereas goblet cells stained blue in the crypt region or dark purple on the villi in infected tissue (B; at 4 dpi). This phenomenon was observed from 1 to 4 dpi in all analyzed sections from infected animals. Using a combined HID-AB staining (C–E), goblet cells in control mice stained black/brown, indicating that they contained sulfated mucins (C; at 7 dpi). In infected mice, goblet cells stained blue, indicating that they predominantly contained sialomucin (D; at 7 dpi). During infection, the percentage of HID-stained goblet cells was significantly decreased at 1 and 7 dpi (E). [a]$P < 0.005$, [‡]$P < 0.001$ vs. control using Student's t test.

Protective capacities of mucins during rotavirus infection

As an initial experiment, we serially diluted mucin isolated from adult mouse large intestine (van Klinken et al., 1999a, 1999b) and assayed viral (Wa strain) infectivity in Caco-2 cells in the presence of mucin (data not shown). The mucin preparation was able to neutralize rotavirus infection in a concentration-dependent manner. The concentration at which viral infectivity was reduced by 50% of this mucin preparation was 10 μg/ml. We determined if the changes in mucin structure during rotavirus infection in neonatal mice had functional implications, in particular on the mucin's capability to inhibit rotavirus infection. We isolated mucins from the small intestines (distal jejunum) of three control and three infected animals per time point at 1, 2, and 4 dpi. These mucin preparations were tested for neutralization of the rotavirus Wa strain (SA-independent) at a concentration of 10 μg/ml. Note that we could not use the mouse EDIM strain for our in vitro experiments since this strain is not cell culture-adapted and does not infect or replicate within the Caco-2 cell line.

Mucins isolated from control and infected mice were able to significantly neutralize Wa rotavirus infection in Caco-2 cells when compared to a similar amount of cells incubated with virus in the absence of mucins. Inhibiting effects of the individual mucin isolates ranged from 78.3% to 27.3% (Table 1). Mucins isolated at 4 dpi from control and infected mice were less efficient in inhibiting rotavirus infection than the respective mucins isolated from control and infected mice at 1 and 2 dpi ($P < 0.05$). At 4 dpi, mucins isolated from infected mice were capable of inhibiting rotavirus infection more efficiently (57.1%) than mucins isolated from control mice (27.3%). We also performed an inhibition study using the rotavirus SA11 (SA dependent) strain and

Table 1
Inhibition of rotavirus infection by mucins isolated from neonatal mice

	Percent inhibition ± SEM		
	1 dpi	2 dpi	4 dpi
Control mice	68.2 ± 2.9	78.3 ± 2.0	27.3 ± 6.1[a]
Infected mice	78.1 ± 2.1	69.1 ± 2.9	57.1 ± 4.1[a]

Mucins were isolated from the small intestines (distal jejunum) of three control and three infected animals per time point at 1, 2, and 4 dpi. Mucin preparations (10 μg/ml) were tested to neutralize infection of rotavirus Wa strain in the Caco-2 cell line. Mean ± SEM percentages inhibition of infection in Caco-2 cells incubated with and without mucins are indicated. Mucins isolated from control and infected mice at 4 dpi were less efficient in inhibiting infection than mucins isolated from control and infected mice at 1 and 2 dpi ([a]$P < 0.05$). The experiments were performed at least 10-fold.
[a] $P < 0.005$, using Student's t test.

obtained similar results as for the Wa strain (data not shown).

All above-mentioned experiments, i.e., the infection of mice and the whole data analysis, except the isolation and subsequent measurement of the mucin inhibitory capacities, were performed twice independently. The results of both experiments were comparable in all aspects.

Discussion

Previous studies demonstrated that rotavirus infection has a profound effect on small intestinal epithelial homeostasis. It was found that rotavirus infection induces apoptosis at the tips of the villi, increases epithelial cell turnover, and hampers enterocyte gene expression (Boshuizen et al., 2003; Collins et al., 1988; Katyal et al., 1999; Osborne et al., 1988; Shepherd et al., 1979; Starkey et al., 1986). Rather than concentrating on enterocyte cell function, we focused on goblet cell homeostasis during rotavirus infection and on the expression of specific gene products that are secreted by these cells. In the present study, we investigated changes in the number of goblet cells, goblet cell migration kinetics, Muc2 and Tff3 mRNA and protein levels, and analyzed the qualitative changes in intestinal mucin glycosylation and sulfation during rotavirus infection in neonatal mice.

Neonatal mice infected with EDIM rotavirus developed diarrhea over a period of 5 days starting at 1-day post infection (dpi). As in other studies, viral infection occurred in two peaks of replication at 1 and 4 dpi (Boshuizen et al., 2003; Osborne et al., 1988; Starkey et al., 1986). Our previous findings demonstrated a severe loss of enterocytes during infection through increased apoptosis (Boshuizen et al., 2003). In these infected mice, the expression of specific enterocyte markers was reduced to only 10–20% of control levels. We found in the duodenum and jejunum that also the number of goblet cells decreased significantly between 1 and 4 dpi. In the ileum, however, infection had little effect on the number of goblet cells. Epithelial cells in infected mice migrated faster up the villus than in control animals, a

process during which the goblet cells accumulated at the tips of the villi. This phenomenon was most prominent in the ileum, but occurred in all of the small intestine. At 4 and 7 dpi, all BrdU-labeled cells that were still present at the villus tips of infected mice were goblet cells. These findings suggest that, at least in the ileum (where we found the highest levels of viral replication), goblet cells, in contrast to enterocytes, are comparatively spared from apoptosis during severe rotavirus infection. The sparing of goblet cells could be a mechanism within the epithelium to uphold their protective function in epithelial defense. This event was also observed in rats during severe intestinal damage induced by the cytostatic drug methotrexate (Verburg et al., 2000).

The potential protective role of goblet cells and their products was further corroborated by our observation that Muc2 and Tff3 levels produced by these cells were maintained or even enhanced during infection. Muc2 mRNA levels per goblet cell in the small intestine were increased during most severe damage and viral replication. Moreover, Tff3 mRNA levels were maintained during rotavirus-induced damage at levels not different from control levels. Interestingly, in the jejunum, total Muc2 protein levels were decreased during viral replication at 1 and 2 dpi, probably due to the observed decrease in the number of goblet cells. However, when the jejunal Muc2 protein levels were considered in relation to their numbers, it appeared that the Muc2 protein production per cell was even increased. This was in contrast to the Tff3 protein levels that were decreased during rotavirus infection. Given the fact that the expression patterns of Muc2 and Tff3 differ in space and time, our results suggest that the Muc2 and Tff3 genes are not coordinately regulated in goblet cells during rotavirus infection. This was also observed in a methotrexate-induced damage and regeneration model in rats (Verburg et al., 2002).

The increased Muc2 mRNA levels observed during infection could be mediated through several mechanisms. Mucus overproduction has long been recognized as an important symptom of respiratory viral infections, but only recently it was shown that mucin is induced as a direct effect of viral double-stranded RNA (dsRNA) on airway epithelial cells (Londhe et al., 2003). The signaling pathways mediating MUC2 transcription by dsRNA were shown to resemble those shown previously to mediate cytokine induction and include the activation of mitogen-activated protein kinases (MAPK) and nuclear factor kappa-B (NF-kappaB) (Londhe et al., 2003). Rotavirus is also able to rapidly activate NF-κB during infection (Rollo et al., 1999). Instead of via dsRNA, however, this activation was shown to be provoked primarily via binding of its outer capsid protein VP4 to the TNFR-associated factor 2 (TRAF2) and activation of TRAF signaling (LaMonica et al., 2001). The above-mentioned pathways are also known to induce the expression of cyclooxygenase 2 (COX2), a key enzyme for the production of prostaglandins (PGs). Our group recently demonstrated that COX2 mRNA and PGE_2 levels are

increased during rotavirus infection in Caco-2 cells and that PGE_2 and COX activity is crucial for rotavirus replication (Rossen et al., 2004). Interestingly, MUC2 protein expression is mediated through PGE_1 and PGE_2 (Willemsen et al., 2003), and moreover, PGE_2 was shown to enhance mucin release via a cAMP-dependent mechanism (Belley and Chadee, 1999).

Bacteria were also shown to induce MUC2 transcription mainly via production of NF-kappaB and various cytokines (Deplancke and Gaskins, 2001; Dohrman et al., 1998; Li et al., 1998; Mack and Sherman, 1991). Among the many (pro-inflammatory) cytokines that stimulate mucin gene expression are IL-1, IL-4, IL-6, IL-9, and tumor necrosis factor alpha (TNF-alpha) (Deplancke and Gaskins, 2001; Van Seuningen et al., 2001 and references therein). It still has to be defined which of these multiple pathways and to what extent are mediating the increased levels of Muc2 mRNA at 1 dpi in this study.

Mucins can roughly be classified into neutral and acidic subtypes. Acidic mucins can be further distinguished by sulfated (sulfomucins) or non-sulfated (sialomucins) groups. In most mammalian species, neutral mucins appear to be the predominant subtype expressed in gastric mucosa, whereas acidic mucins dominate in the small and large intestine (Sheahan and Jervis, 1976). During rotavirus infection, we found a reduction in the number of goblet cells producing sulfated mucins. Studies of SA11 rotavirus infection in rats also showed a reduction in the number of sulfated mucin-containing cells (Guerin-Danan et al., 2001). SA11-infected rats fed with milk fermented by *Lactobacillus casei* had less diarrhea and the number of sulfated mucin-containing cells was comparable to controls (Guerin-Danan et al., 2001). We observed that the small intestinal mucins were particularly less sulfated during or after the two replication peaks. At these time points, sulfated mucins could have been preferentially secreted or the mucin molecules could be undersulfated as has been observed in patients with ulcerative colitis or in a rat model for colitis (Jass and Roberton, 1994; Renes et al., 2002a; van Klinken et al., 1999a, 1999b). Sulfate content of mucins is thought to present resistance to (bacterial) enzymatic degradation of the mucus layer (Nieuw Amerongen et al., 1998; Robertson and Wright, 1997) and therefore the decrease in sulfated mucins could influence the consistency of the mucus layer. Changes in mucin sulfation particularly will be of relevance in the colon where sulfate-reducing bacteria reside. It will therefore be of special interest to determine whether sulfation is also reduced in the colon during rotavirus infection.

AB-PAS staining suggested that the fraction of acidic mucins was strongly increased during rotavirus infection in mice. Since we found that the number of goblet cells producing sulfated mucins was decreased, these acidic mucins most probably embody heavily sialylated mucins. Several studies have reported that glycosylation and sialylation of mucins change during bacterial or parasite infections (Davril et al., 1999; Holmen et al., 2002). Davril

et al. reported that in patients who were severely infected with *Pseudomonas aeruginosa*, mucins isolated from bronchial mucosa had a higher sialic acid content than mucins from non-infected patients (Davril et al., 1999). The authors also concluded that bacterial infection influences the expression of sialyltransferases and fucosyltransferases in the human bronchial mucosa. In vitro, cytokines have been implicated in the sialylation of mucins (Delmotte et al., 2001). In particular, TNF-alpha was shown to upregulate mRNA levels of sialyl- and of fucosyl-transferases and increase the sialylation of mucins in a human respiratory gland cell line. Interestingly, TNF-alpha levels were found increased in rotavirus-infected children and increased levels correlated with the severity of the symptoms (Jiang et al., 2003).

Mucins of various origins have been implicated in the inhibition of rotavirus infection (Chen et al., 1993; Newburg et al., 1998; Peterson et al., 2001; Willoughby and Yolken, 1990; Yolken et al., 1987, 1994). Mucins are thought to present a barrier for infection through direct interaction with the virus, thereby preventing virus-cell attachment (Chen et al., 1993; Yolken et al., 1987). The changes in mucin sulfation and especially sialylation during rotavirus infection in mice suggested that these mucins would have an altered competence to inhibit rotavirus infection. Mucins isolated at 4 dpi from control and infected mice were less efficient in inhibiting rotavirus infection than mucins isolated from control and infected mice at 1 and 2 dpi. Our data therefore suggest that, as was previously found (Chen et al., 1993), age-dependent differences in mucin composition alter the inhibitory capabilities of small intestinal mucins. Mucins isolated from infected mice at 4 dpi were more efficient in neutralizing rotavirus infection than mucins isolated from control mice at 4 dpi. This implies that the increase in sialylated mucins in infected animals at 4 dpi results in the increased protective capacity compared to controls at this time point. This observation could represent part of an innate defense mechanism to remove pathogens. The specific relationship between mucin oligosaccharide composition alterations and susceptibility to infection, however, is still the subject of ongoing investigations. Additionally, more experiments have to be performed in order to substantiate the possible role of the rotavirus-increased occurrence of acid mucins and their role in the defense against rotavirus infection.

In conclusion, our data show that during rotavirus infection, goblet cells, in contrast to enterocytes, are spared from apoptosis in the ileum, Muc2 levels are up-regulated, and mucin structure is changed. This suggests that mucins and in particular Muc2 play an important role in the active defense against rotavirus infection. The data further imply that age-dependent differences in mucin composition and increased occurrence of acidic (most likely sialylated) mucins alter the inhibitory capabilities of mucins isolated from the small intestine. This suggests that posttranslational modification in glycosylation of mucins during infection

may affect specific epithelial barrier function against intestinal pathogens.

Materials and methods

Animals

Pregnant dams were obtained from Harlan (Zoetermeer, The Netherlands). Dams, either with their control or inoculated litters, were housed in micro-isolator cages under negative pressure in a specified pathogen-free environment. Rodent, chicken protein-free chow (9605/9608; Harlan Teklad TRM (A)) and deionized water were autoclaved and provided ad libitum until the end of the experiment. All dams were rotavirus antibody negative as measured by enzyme-linked immunosorbent assay (ELISA, as described below). All the experiments were performed with the approval of the Animal Studies Ethics Committee of the National Institute of Public Health and the Environment.

Virus inoculations and subsequent animal-handling procedures

The Epizootic Diarrhea of Infant Mice (EDIM) mouse rotavirus strain was obtained from Dr. R. Ward, Children's Hospital Research Foundation, Cincinnati, USA (McNeal et al., 1995). This strain is an unpassaged virus isolated directly from the stools of ill mice. Six-day-old BALB/c mice were inoculated intra-gastrically with 2×10^4 focus forming units (ffu) of the EDIM rotavirus strain, as described previously (Boshuizen et al., 2003). To analyze epithelial proliferative kinetics, 30 mg/kg bodyweight 5-bromo-2′-deoxyuridine (BrdU; Sigma, St Louis, USA) was injected intraperitoneally 5 min before inoculation. Control mice were mock infected through inoculation with PBS. After 6 h and at days 1, 2, 4, 7, and 14, three infected and three control mice were sacrificed per time point. The infection of mice and all subsequently experiments and data analysis, except the experiment measuring the mucin inhibitory capacities, were performed twice independently. The results of the independent experiments were comparable in all aspects. Segments of duodenum (i.e., proximal 2 cm of the small intestine), jejunum (i.e., 2 cm intestine taken from the anatomic middle of the small intestine), and ileum (i.e., distal 2 cm of the small intestine) were rinsed in PBS and fixed for 4 h in 4% (wt/vol) paraformaldehyde (Merck, Darmstadt, Germany) dissolved in PBS. The segments were subsequently dehydrated through a graded series of ethanol and xylene (Merck), and embedded in Paraplast Plus (Sherwood Medical, Den Bosch, The Netherlands) as previously described (Boshuizen et al., 2003; Verburg et al., 2000). In addition, adjacent segments of the jejunum and ileum were dissected and snap frozen in liquid nitrogen and stored at −80 °C for RNA and protein isolation.

Histology

Paraffin-embedded tissues were sectioned (5 μm), deparaffinized with xylene (Merck), and rehydrated in graded ethanol solutions as previously described (Boshuizen et al., 2003; Renes et al., 2002b). Histochemical staining was performed by using Alcian blue (AB) at pH 2.5 followed by periodic acid-Schiffs reagent (PAS), or high iron diamine (HID) followed by AB at pH 2.5 as previously described (Makkink et al., 2002). The numbers of goblet cells per villus were counted after AB-PAS staining in 20-well-oriented crypt-villus units of the duodenum, jejunum, and ileum, respectively, per animal per time point until day 7 dpi. The numbers of HID-positive goblet cells were counted in 10-well-oriented crypt-villus units per animal in the ileum per animal per time point until day 7 dpi.

Immunohistochemistry

Deparaffinized and rehydrated sections were incubated overnight at 4 °C using the primary mouse antibody anti-BrdU (1:250; Boehringer, Mannheim GmbH, Mannheim, Germany), caspase-3 (1:500; Cell Signaling Technology, Beverly, USA), or a rabbit hyperimmune serum raised against SA11 rotavirus particles (1:1500; Boshuizen et al., 2003). Immunohistochemistry was carried out as described previously (Boshuizen et al., 2003; Renes et al., 2002b). Sections that had been incubated with the anti-BrdU antibody were washed with PBS and subsequently treated with AB at pH 2.5.

In situ hybridization and probe preparation

Non-radioactive in situ hybridization was performed using the method as previously described (Lindenbergh-Kortleve et al., 1997; Renes et al., 2002b). Digoxigenin (DIG)-11-UTP-labeled RNA probes were prepared according to the manufacturer's protocol (Boehringer) using T3, T7, or SP6 RNA polymerase. The following probes were used: a 244-bp murine Muc2 probe (van Klinken et al., 1999a, 1999b) and a 901-bp VP4 fragment (EDIM strain) ligated in pBluescript (see below). Transcripts longer than 450 bp were hydrolyzed in 80 mM NaHCO₃ and 120 mM Na₂CO₃, pH 10.2 to obtain probes of various lengths ≤450 bp (Cox et al., 1984).

Quantitation of mRNA

Total RNA was isolated from frozen small intestinal segments using Trizol reagent (Gibco-BRL, Gaithersburg, MD, USA) following the manufacturer's protocol. Integrity of the RNA was assessed by visual analysis of the 28S and 18S ribosomal RNAs after electrophoresis and staining with ethidium bromide. Subsequently, 1 μg of total RNA was dot-blotted on Hybond-N⁺ (Amersham, Bucks, England) using a vacuum-operated manifold (BioDot, BioRad, Hercules,

CA). All blots were hybridized using specific ^{32}P-labeled cDNA probes as described previously (Verburg et al., 2000). To correct for the amount of RNA that was spotted, hybridized signals were standardized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using a 1.4-kb human GAPDH probe (Boshuizen et al., 2003; Verburg et al., 2000). Hybridization signals were measured through autoradiography using a PhosphorImager and ImageQuant software (Molecular Imaging, Sunnyvale, CA, USA). The following probes were used: the pBluescript cDNA probes that were used were the 244-bp murine Muc2 probe (van Klinken et al., 1999a, 1999b), a 438-bp rat Tff3 probe (Suemori et al., 1991), or a 900-bp VP4 probe. For construction of the VP4 probe, RT-PCR was performed on RNA isolated from EDIM-infected neonatal mice. VP4 was cloned using the primers TATACCATGGCTTCACTCATT-TATAGAC and TTGGTAGTTCGCTGGTTTGAA, based on the EDIM VP4 coding sequence accession number AF039219 (GenBank). PCR fragments were TA-cloned into the PCR 2.1 vector (Invitrogen, Breda, The Netherlands) and sequenced.

Quantitation of rotavirus protein

Proteins were isolated from frozen segments by homogenization of the tissue in 500 µl (Tris-) homogenization buffer containing Triton X-100 (BDH, Poole, England), 1% SDS, and various protease inhibitors as previously described. (Boshuizen et al., 2003; Renes et al., 2002a). Protein concentration was measured using a bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, IL, USA). Bovine serum albumin was used as standard. To quantify rotavirus structural protein contents, protein homogenates were dot-blotted on nitrocellulose (Nitran; Schleier and Schuell, Dassell, Germany). The blots were blocked for 1 h with blocking buffer (50 mM Tris–HCl, pH 7.8, 5% (wt/vol) nonfat dry milk powder; Lyempf, Kampen, The Netherlands), 2 mM $CaCl_2$, 0.05% (vol/vol) Nonidet P40 (BDH), and 0.01% (vol/vol) antifoam (Sigma, St Louis, USA), and incubated for 18 h with anti-mouse Muc2 serum (1:500) (van Klinken et al., 1999a, 1999b) or anti-rat Tff3 serum (1:1500), a generous gift from Daniel K. Podolsky (Suemori et al., 1991). After being washed in blocking buffer, the blots were incubated with ^{125}I-labeled protein A (specific activity 33.8 mCi/mg; Amersham) for 2 h. Bound ^{125}I-labeled protein A was then detected and the elicited signals were quantified using a PhosphorImager and ImageQuant software (Molecular Imaging).

Isolation of mucins

Mouse colonic mucin was isolated and prepared from mucosal scrapings, as described previously (van Klinken et al., 1999a, 1999b). Mucins from the small intestine (distal jejunum) of rotavirus-infected or control mouse neonatal pups were isolated with the following procedure. Whole tissue segments were homogenized at 4 °C in 6 M guanidium hydrichloride (Sigma). The mucins were reduced by addition of 100 mM dithiothreitol (Sigma) to enhance solubility. Sulfhydride groups were carboxymethylated through addition of 250 mM iodoacetamide (Sigma) and stirring for 24 h at 4 °C. Mucins were then fractionated by gelfiltration on a sepharose column (Bio-Gel A-50m; Bio-Rad, Veenendaal, The Netherlands). Fractions were analyzed for purity and mucin content by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) followed by periodic acid-Schiff (PAS) staining. Mucin-containing fractions were pooled and dialyzed extensively against distilled water at 4 °C. Mucin preparations were quantified as described previously by a hexose assay using orcinol (Sigma) and galactose/fucose (3:2) as standard (Tytgat et al., 1994).

Cell culture, rotavirus infection, and immunofluorescence assay to measure the protective capacities of mucins

Human colonic adenocarcinoma Caco-2 cells (ATCC, Manassas, VA, USA) were grown and maintained in 75-cm^2 tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) with glutamax-1, pyridoxine, sodium pyruvate, and 4.5 g/l glucose, supplemented with 10% fetal calf serum (Integro, Zaandam, The Netherlands), 100 U/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma), and non-essential amino acids (Bio Whittaker Europe, Verviers, Belgium) at 37 °C, 5% CO_2 in a humidified atmosphere. One day prior to infection, Caco-2 cells were seeded on 10-well heavy Teflon-coated microscopic slides (7 mm, Nutacon, Leimuiden, The Netherlands) at 10^4 cells/well as described previously (Rossen et al., 2004). One hour prior to infection, the Caco-2 cells were rinsed 3 times in serum-free DMEM and incubated together with diluted purified mucins. Meanwhile, the human rotavirus strain Wa was activated using 10 mg/ml trypsin (Sigma) at 37 °C for 1 h. Rotavirus was diluted to 2×10^2 ffu/ml (corresponding to about 100 ffu per well) in serum-free DMEM containing the diluted mucins. Cells were infected with rotavirus at 37 °C, 5% CO_2 in a humidified atmosphere. After 15 h of incubation, infection was terminated using ice-cold methanol and cells were stored for 10 min at −20 °C. The microscopic slides with the methanol-fixed infected Caco-2 cells (Rossen et al., 2004) were rinsed 3 times in PBS and then incubated for 90 min at RT with rabbit hyperimmune serum raised against SA11 rotavirus particles (1:1600) (Boshuizen et al., 2003; Rossen et al., 2004). Slides were then rinsed again 3 times and incubated with a secondary anti-rabbit Texas Red-conjugated antibody (1:200; Jackson Immuno Research Laboratories Inc., West Grove, PA, USA). Slides were mounted with a solution containing Mowiol (Sigma), 2.5% DABCO (1,4-Diazabicyclo[2.2.2]octane, Sigma), and 0.5 µg/ml 4′,6′-diamidino-2-phenylindole dihydrochloride/hydrate (DAPI, Sigma) (Heimer and Taylor,

1974). Fluorescence was observed using a Nikon Eclipse 800 microscope and the number of foci was determined at 200× magnification.

Statistical analysis

Statistical analysis of all data from control and infected groups was performed using Student's *t* test for unpaired data (two-tailed). To compare three or more groups, data were analyzed by ANOVA followed by an unpaired *t* test. Data were expressed as the mean ± SEM and *P* values of ≤0.05 were considered statistically significant.

Acknowledgments

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