Journal of General Virology (2007), **88**, 2064–2072 **DOI** 10.1099/vir.0.82618-0

Induction of nitric oxide synthase by rotavirus enterotoxin NSP4: implication for rotavirus pathogenicity

Mohamed A. Borghan,[1]† Yoshio Mori,[2] Abu-Baker El-Mahmoudy,[3] Naoto Ito,[1] Makoto Sugiyama,[1] Tadashi Takewaki[3] and Nobuyuki Minamoto[1]

Correspondence
Naoto Ito
naotoito@gifu-u.ac.jp

[1]Laboratory of Zoonotic Diseases, Department of Veterinary Medicine, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

[2]Research Institute for Microbial Diseases, Osaka University, Japan

[3]Laboratory of Physiology, Department of Veterinary Medicine, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

Rotavirus non-structural protein (NSP) 4 can induce aqueous secretion in the gastrointestinal tract of neonatal mice through activation of an age- and Ca^{2+}-dependent plasma membrane anion permeability. Accumulating evidence suggests that nitric oxide (NO) plays a role in the modulation of aqueous secretion and the barrier function of intestinal cells. This study investigated transcriptional changes in inducible NO synthase (iNOS), an enzyme responsible for NO production, after rotavirus infection in mice and after treatment of intestinal cells with NSP4. Diarrhoea was observed in 5-day-old CD-1 mice from days 1 to 3 after inoculation with 10^7 focus-forming units of different rotavirus strains. Ileal iNOS mRNA expression was induced as early as 6 h post-inoculation, before the onset of clinical diarrhoea in infected mice, and was upregulated during the course of rotavirus-induced diarrhoea. *Ex vivo* treatment of ilea excised from CD-1 suckling mice with NSP4 resulted in upregulation of ileal iNOS mRNA expression within 4 h. Furthermore, NSP4 was able to induce iNOS expression and NO production in murine peritoneal macrophages and RAW264.7 cells. The specificity of NSP4 inducibility was confirmed by the inhibitory effect of anti-NSP4 serum. Using a series of truncated NSP4s, the domain responsible for iNOS induction in macrophages was mapped to the reported enterotoxin domain, aa 109–135. Thus, rotavirus infection induces ileal iNOS expression *in vivo* and rotavirus NSP4 also induces iNOS expression in the ileum and macrophages. Together, these findings suggest that NO plays a role in rotavirus-induced diarrhoea.

Received 4 October 2006
Accepted 6 March 2007

INTRODUCTION

Rotavirus, the most common mammalian enteric viral pathogen, exclusively infects differentiated epithelial cells of intestinal villi in infants and young animals (Kapikian & Chanock, 1990). Rotavirus infection in mammals is associated with blunting of the intestinal villi and mild infiltration of mononuclear cells in lamina propria (Greenberg *et al.*, 1994). Rotavirus non-structural protein 4 (NSP4) and a synthetic peptide corresponding to aa 114–135 of NSP4 ($NSP4_{114–135}$) have been shown to cause diarrhoea in suckling mice (Ball *et al.*, 1996; Mori *et al.*, 2002). NSP4 as a viral enterotoxin induces age- and Ca^{2+}-dependent movement of halide ions across the plasma membrane in the initial cyclic nucleotide-independent pathway (Dong *et al.*, 1997; Morris *et al.*, 1999). Apart from the direct action of NSP4 on the mucosa at the onset of rotavirus diarrhoea, secondary mechanisms triggered by rotavirus replication and/or NSP4 are thought to sustain the diarrhoeal response. These mechanisms include mucosal destruction (Davidson & Barnes, 1979; Greenberg *et al.*, 1994), villus ischaemia (Osborne *et al.*, 1991), alteration of paracellular permeability and of expression of digestive enzymes (Jourdan *et al.*, 1998; Dickman *et al.*, 2000), and activation of the enteric nervous system (ENS) (Lundgren *et al.*, 2000). The latter may explain how comparatively few infected cells at the villus tips can induce secretion of electrolytes and water from intestinal crypts. However, it is not known how rotavirus infection activates the ENS. Nevertheless, it is now recognized that the intestinal epithelium can release a wide range of biologically active compounds such as cytokines, prostaglandins and nitric

†Present address: Center for Cancer Rersearch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA.

oxide (NO) upon exposure to bacteria and viruses (Jung *et al.*, 1995; Salzman *et al.*, 1998; Rollo *et al.*, 1999). Such mediators may influence intestinal fluid secretion either directly by acting on intestinal epithelial cells or indirectly through activation of the ENS.

NO is generated from L-arginine by a family of NO synthases (NOSs) (Nathan & Xie, 1994). There are three isoforms of NOS, named according to the cell type or to the condition under which they were first identified: endothelial NOS (eNOS), neuronal NOS (nNOS) and macrophage or inducible NOS (iNOS) (Nathan & Xie, 1994). The iNOS isoform, in contrast to eNOS and nNOS, is not expressed constitutively, but can be induced in a wide variety of cells by various stimulators such as bacterial lipopolysaccharide (LPS), gamma interferon and interleukin-1 (Nathan & Xie, 1994), leading to the generation of a large amount of NO. In the gastrointestinal tract, NO has been shown to regulate intestinal motility (Bult *et al.*, 1990; Hoffman *et al.*, 1997) and mucosal permeability (Kubes, 1992). However, accumulating evidence indicates that NO acts as a modulator of intestinal ion transport via either its activation of the ENS (Wilson *et al.*, 1993; Izzo *et al.*, 1998; Rhee *et al.*, 2001) or its direct action on the intestinal epithelium (MacNaughton, 1993; Stack *et al.*, 1996; Rolfe & Milla, 1999; Resta-Lenert & Barrett, 2002). However, direct involvement of NO in diarrhoea associated with intestinal pathogens, including rotavirus, remains to be shown.

A deeper understanding of how the host responds to rotavirus infection and to the actions of key viral proteins is important for elucidating the basis of viral pathogenicity. In the present study, we demonstrated that rotavirus infection can stimulate iNOS expression in the murine ileum. iNOS expression was also shown to be upregulated in the murine ileum and in macrophages upon exposure to NSP4. Furthermore, the enterotoxin domain of NSP4 was shown to be able to induce iNOS expression. These findings suggest that NO plays a role in rotavirus-induced diarrhoea.

METHODS

Cells and viruses. MA104 cells were grown in Eagle's minimal essential medium (EMEM) supplemented with 10 % fetal bovine serum (FBS). Murine macrophage-like RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FBS, 2 mM L-glutamine, 10 mM HEPES, 100 U penicillin ml^{-1} and 100 μg streptomycin ml^{-1}. Peritoneal macrophages were isolated from adult CD-1 mice (Charles River, Yokohama, Japan) by peritoneal lavage and maintained in supplemented DMEM. Rotavirus strains simian SA-11, murine EW and avian PO-13 were propagated in MA104 cells in the presence of trypsin as described elsewhere (Minamoto *et al.*, 1988). To prepare inactivated rotavirus, strain SA-11 was mixed with 4′-aminomethyl-4,5′,8-trimethyl psoralen (40 μg ml^{-1}) and incubated on ice under UV light for 50 min (Groene & Shaw, 1992). The psoralen-inactivated SA-11 virus (PI-SA11) was characterized by failure to infect MA104 monolayers as determined by a focus assay 24–96 h post-inoculation (p.i.) and blind passages.

Rotavirus inoculations and diarrhoea score. Five-day-old CD-1 mice were inoculated orally with 10^7 focus-forming units (f.f.u.) of simian SA-11, murine EW, avian PO-13 or PI-SA11. Mock inoculations were performed using EMEM without additives. Mice were monitored every day for 4 days. Diarrhoea was noted and a four-point qualitative scale was applied as follows: 1, normal brown formed stool; 2, yellow pasty stool; 3, yellow mixed liquid and solid stool; and 4, entirely liquid stool. A score of 2 or above was considered to be diarrhoea (Mori *et al.*, 2001). For mRNA analysis, 4 cm long ileal segments were collected from control and infected mice, rinsed with supplemented DMEM and snap-frozen in liquid nitrogen.

NSP4s and culture conditions. Full-length glutathione *S*-transferase (GST)-fused NSP4 (GST–NSP4) from the avian PO-13 strain, four GST-fused truncated PO-13 NSP4s corresponding to aa 86–169 (GST–$NSP4_{86–169}$), aa 109–169 (GST–$NSP4_{109–169}$), aa 86–135 (GST–$NSP4_{86–135}$) and aa 86–169 truncated at aa 112–133 (GST–$NSP4_{86–169\Delta112–133}$), GST-fused VP8 (GST–VP8) from the avian PO-13 strain and GST were expressed in *Escherichia coli* as described previously (Mori *et al.*, 2002). Briefly, overnight cultures of *E. coli* DH5α strain transformed with recombinant pGEX-2T were diluted in fresh Luria–Bertani medium and grown to an OD_{600} of 0.3–0.4. Gene expression was induced by the addition of IPTG (Roche Diagnostics) to 0.1 mM and cultures were incubated for a further 12 h. Bacteria were pelleted by centrifugation at 4000 ***g*** for 10 min at 4 °C and resuspended in a sonication buffer [1 M Tris/HCl (pH 8.1), 0.5 M EDTA, 5 M NaCl] supplemented with 5 mM DDT and 1 % Triton X-100. Cell suspensions were then sonicated on ice with five short bursts of 10 s followed by intervals of 10 s. Debris was removed by centrifugation at 18 000 ***g*** for 30 min at 4 °C. Clarified cell lysates were passed through a GST 4B column (Amersham Pharmacia Biotech) three times. The column was washed three times and bound proteins were eluted with glutathione elution buffer [10 mM reduced glutathione in 50 mM Tris/HCl (pH 8.0)]. Proteins were then concentrated by ultrafiltration using Ultrafree-MC filtration devices with a 10 kDa nominal molecular mass limit (Millipore). Protein concentrations were determined using a Bio-Rad protein assay. Expression and purity of the recombinant proteins were determined by SDS-PAGE followed by Coomassie brilliant blue staining and Western blot analyses (data not shown, Borgan *et al.*, 2003). Purified proteins were shown to be free of detectable endotoxin as determined by a *Limulus* amoebocyte lysate kit (Sigma). Production of polyclonal guinea pig anti-PO-13 NSP4 serum has been described elsewhere (Borgan *et al.*, 2003). Peritoneal macrophages or confluent RAW264.7 cells were exposed to culture medium containing tenfold serial dilutions of GST–NSP4 (from 100 to 0.1 pmol), GST–VP8 (from 200 to 0.2 pmol), GST (from 200 to 0.2 pmol) or truncated GST–NSP4s (from 200 pmol to 0.2 pmol). LPS from *E. coli* 0127 : B8 (Sigma) was used as a positive control. For an antibody-dependent inhibition experiment, GST–NSP4 was pre-treated with polyclonal anti-PO-13 NSP4 guinea pig serum or with normal guinea pig serum for 1 h at 37 °C prior to cell treatment.

mRNA analysis. Total RNA was extracted from macrophages treated with GST–NSP4 for 4 h or from frozen ileal segments using ISOGEN reagent (Nippon Gene). Contaminant genomic DNA was removed by DNase I (Takara). First-strand cDNA was synthesized from total RNA (5 μg) using an anchored $oligo(dT)_{23}$ primer (Sigma) and Ready-To-Go You-Prime First-Strand beads (Amersham Pharmacia Biotech). cDNA (1 μl) was used as template for PCR amplification of iNOS message with *Ex-Taq* DNA polymerase (TaKaRa) for 35 cycles of 95 °C for 30 s, 68 °C for 60 s and 72 °C for 60 s. The iNOS cDNA was amplified using the following primers: sense, 5′-AGTGGGCCGAAGGATGGGCCTGGAG-3′, and anti-sense, 5′-GTCTCACAGGCTGCCCGGAAGGTTTG-3′. The β-actin gene was used as an internal control. β-Actin primers and PCR conditions were as described

elsewhere (Perrin *et al.*, 1996). PCR products were resolved on a 1.5 % agarose gel containing ethidium bromide and visualized under UV light. PCR products were analysed further by DNA sequencing. Relative amounts of PCR products were determined by densitometry using NIH IMAGE 1.62 software and the ratio of iNOS mRNA to β-actin mRNA was calculated.

Western blot analysis. Macrophages treated for 24 h with GST–NSP4 or truncated GST–NSP4s were lysed with 100 µl of lysis buffer [25 mM Tris/HCl (pH 7.4) containing 150 mM Nacl, 1 % NP-40, 1 mM DTT and 50 µg ml^{-1} each of aprotinin, PMSF and leupeptin]. Soluble proteins (25 µg per lane) were separated by SDS-PAGE on 10 % gels and transferred to Immobilon PVDF transfer membranes (Millipore) by electroblotting. Membranes were then blocked overnight at 4 °C using PBS containing 0.1 % Tween 20 and 5 % non-fat dried milk. iNOS was detected by polyclonal antibodies to murine iNOS (diluted 1 : 1000; Sigma). An internal control, α-tubulin, was detected by monoclonal antibodies to murine α-tubulin (diluted 1 : 4000; Sigma). Signals were detected using Western Blot Chemiluminescence Reagent Plus (NEN Life Science Products) and a luminescence image analyser (LAS-1000; Fuji Film).

Measurement of NO concentration. The concentrations of the oxidized product of NO, nitrite (NO^{2-}), in the culture media were determined using Griess reagent (Green *et al.*, 1982).

***Ex vivo* treatment of ileum by GST–NSP4.** Four-day-old CD-1 mice were sacrificed under diethyl ether anaesthesia and 4 cm long ileal segments were harvested, flushed with DMEM and maintained in DMEM containing 2 % FBS and 2 µg gentamicin ml^{-1}. After incubation for 1 h at 37 °C, the ends of the ileal segments were tied off and 50 µl of GST–NSP4, GST–VP8 or GST was inoculated. Ileal segments were then incubated for a further 4 h, followed by total RNA extraction and RT-PCR analysis as described above.

RESULTS

Induction of iNOS mRNA expression in the murine ileum during the course of rotavirus-induced diarrhoea

The mouse model of rotavirus infection and diarrhoea is well characterized and has been used for many studies of pathogenesis and immunity (Greenberg *et al.*, 1994; Mori *et al.*, 2001). We first sought to determine whether iNOS induction also occurred during the course of rotavirus-induced diarrhoea. Five-day-old CD-1 mice were inoculated orally with 10^7 f.f.u. simian SA-11, murine EW or avian PO-13, and diarrhoea was monitored daily as described in Methods. At 42 h p.i., when the severity of diarrhoea was greatest in suckling mice (data not shown), ileal segments were excised from the mice and subjected to RT-PCR analysis. β-Actin mRNA was used as an internal standard control for RNA comparisons. Marked induction of iNOS mRNA expression was found in the infected mice but not in the control mice (Fig. 1). Nucleotide sequence analysis of the PCR products confirmed the amplification of authentic mouse iNOS. Induction of ileal iNOS mRNA expression by different rotavirus strains indicated that upregulation of iNOS mRNA expression is a common host response to rotavirus diarrhoea.

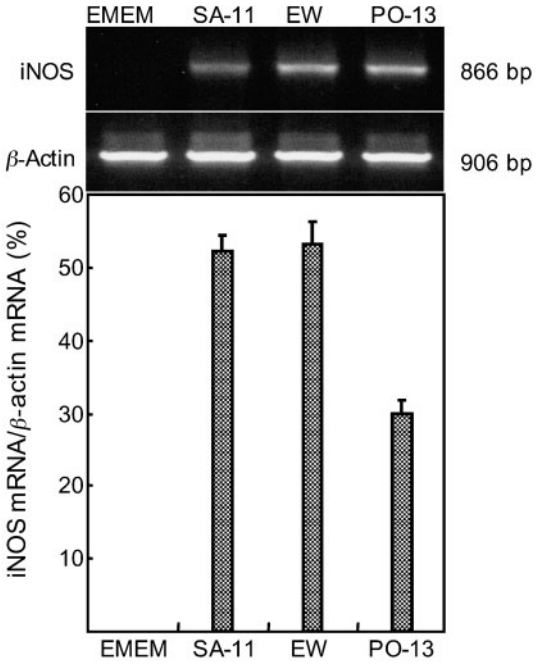

Fig. 1. Induction of iNOS mRNA by rotavirus infection. Five-day-old CD-1 mice were inoculated orally with 10^7 f.f.u. avian rotavirus PO-13, simian rotavirus SA-11 or murine rotavirus EW. Control mice were mock-inoculated with EMEM without additives. At 42 h p.i., when diarrhoea had peaked, 4 cm long ileal segments were harvested from mice and analysed for iNOS mRNA expression by semi-quantitative RT-PCR. PCR products were stained with ethidium bromide and the intensity of stained bands was analysed using NIH IMAGE 1.62 software. The upper panels show a representative RT-PCR analysis of iNOS mRNA (866 bp) and control β-actin mRNA (906 bp). The lower panel shows the ratios of ileal iNOS mRNA to standard ileal β-actin mRNA calculated from the relative intensities. Results are means ± SD from three independent experiments.

Kinetics of ileal iNOS induction by rotavirus infection

To determine the kinetics of ileal iNOS induction, 5-day-old CD-1 mice were inoculated with 10^7 f.f.u. strain SA-11 and ileal segments were collected every 6 h up to 78 h p.i., a time course that enables investigation of not only biological events during the diarrhoea but also pre- and post-diarrhoeal events. The results showed that expression of iNOS mRNA was induced as early as 6 h p.i., with peak values between 12 and 36 h p.i., followed by a progressive decrease after 36 h p.i. (Fig. 2). Expression of iNOS mRNA was markedly upregulated before the onset of clinical diarrhoea and was maintained at an elevated level for almost the entire course of diarrhoea. iNOS mRNA expression was, however, downregulated when diarrhoea was still at its peak (42 and 48 h p.i.), reaching an undetectable level by 48 h p.i. (Fig. 2).

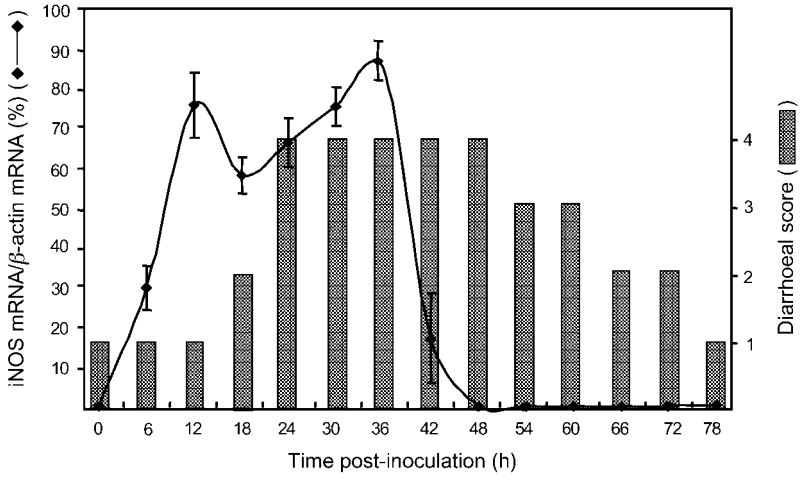

Fig. 2. Kinetics of ileal iNOS elevation after rotavirus infection. Five-day-old CD-1 mice were inoculated orally with 10^7 f.f.u. simian rotavirus SA-11. Ileal segments were collected every 6 h and iNOS mRNA was determined by RT-PCR. PCR products were stained with ethidium bromide and the intensity of stained bands was analysed using NIH IMAGE 1.62 software. The ratios of ileal iNOS mRNA levels to standard ileal β-actin mRNA levels were calculated from relative intensities and are indicated by a line. Results are expressed as means ± SD (n=3). Columns indicate the diarrhoeal score. Diarrhoea was scored as described in Methods.

Effect of virus replication on ileal iNOS mRNA expression

To determine whether induction of iNOS mRNA expression is dependent on rotavirus replication, we used psoralen-inactivated rotavirus strain SA-11 (PI-SA11) to inoculate suckling mice. Psoralen-inactivated rotaviruses maintain their ability to interact with monoclonal antibodies directed to surface proteins and to bind to their receptors, but these viruses are not able to replicate (Groene & Shaw, 1992). Others have also confirmed that rotaviruses maintain their antigenic integrity after UV/psoralen inactivation (McNeal *et al.*, 1999; Londrigan *et al.*, 2003; Holloway & Coulson, 2006).Virus inactivation was confirmed by an immunofluorescent assay and two blind passages in cultured cells (data not shown). Identical amounts of PI-SA11 and untreated SA-11, corresponding to 10^7 f.f.u., were administrated orally to 5-day-old mice. Diarrhoea was monitored daily in order to assess whether the inactivated virus had any residual infectivity. In contrast to live viruses, no diarrhoea was observed in mice inoculated with PI-SA11 up to 5 days p.i., confirming that PI-SA11 was replication incompetent. In order to assess ileal iNOS mRNA expression, ilea were collected at 18 and 24 h p.i. and analysed by RT-PCR. Interestingly, in contrast to live virus, PI-SA11 was unable to induce ileal iNOS mRNA expression (Fig. 3a), indicating that ileal iNOS mRNA expression is associated with rotavirus replication.

Ileal iNOS mRNA expression in response to GST–NSP4 exposure

Studies of the specific roles played by individual rotavirus genes *in vivo* have been extremely limited due to the lack of a reverse genetics system for rotavirus and the current impracticality of some of the presently available approaches such as RNA interference. To determine whether NSP4 was capable of inducing ileal iNOS mRNA expression, we expressed PO-13 NSP4 fused to GST using an *E. coli* expression system and used it for the following experiments. This NSP4 has been well characterized by us (Mori *et al.*, 2002; Borgan *et al.*, 2003) and is known to have strong enterotoxin activity, comparable to that of mammalian rotavirus NSP4 (Mori *et al.*, 2002). Ileal segments excised from 4-day-old mice were maintained in supplemented DMEM and treated with GST–NSP4 (1000 pmol), GST–VP8 (2000 pmol) or GST (2000 pmol). Ileal segments

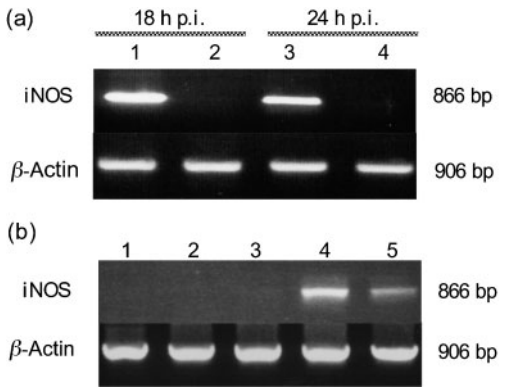

Fig. 3. Effect of viral inactivation on ileal iNOS induction and induction of iNOS mRNA in murine ileum with NSP4. (a) Five-day-old CD-1 mice were inoculated orally with 10^7 f.f.u. live rotavirus, strain SA-11 (lanes 1 and 3), or psoralen/UV-inactivated strain SA-11 (lanes 2 and 4). Ileal segments were harvested at 18 and 24 h p.i. Ileal iNOS expression was then assessed by RT-PCR. The results are representative of two separate experiments. (b) Ileal segments were collected from 4-day-old mice and incubated at 37 °C for 1 h. Ileal segments were then inoculated with GST (2000 pmol, lane 2), GST–VP8 (2000 pmol, lane 3) or GST–NSP4 (1000 pmol) pre-treated with normal guinea pig serum (lane 4) or with anti-PO-13 NSP4 guinea pig serum (lane 5) for 4 h at 37 °C. Untreated ileal segments were used as a control (lane 1). Induction of ileal iNOS mRNA expression was assessed by RT-PCR. The data shown are representative of findings in separate distinct experiments.

were then incubated at 37 °C for 4 h followed by total RNA extraction and RT-PCR analysis. Expression of ileal iNOS mRNA was markedly induced after GST–NSP4 treatment but not after GST–VP8 or GST treatment (Fig. 3b). Pre-treatment of GST–NSP4 with polyclonal guinea pig anti-PO-13 NSP4 serum significantly reduced the expression of ileal iNOS mRNA (Fig. 3b, lane 5), indicating the specificity of GST–NSP4 inducibility. These results showed that rotavirus NSP4 directly induces the expression of ileal iNOS mRNA.

iNOS expression and NO production by macrophages in response to GST–NSP4 exposure

iNOS expression and NO production in response to rotavirus NSP4 were assessed in murine peritoneal macrophages and RAW264.7 cells. In general, iNOS can readily be induced in macrophages by a variety of stimulators such as bacterial LPS and cytokines (MacMicking *et al.*, 1997). When peritoneal macrophages were exposed to tenfold serial dilutions of GST–NSP4 (from 100 to 1 pmol) for 4 h, expression of iNOS mRNA was induced in a dose-dependent manner (Fig. 4a, lanes 3–5). The expression of iNOS mRNAs was undetectable in macrophages treated with 0.1 pmol GST–NSP4 (Fig. 4a, lane 6). Exposure of peritoneal macrophages to 200 pmol GST or GST–VP8 did not induce expression of iNOS mRNA (Fig. 4a, lanes 7 and 8). These results were further confirmed by analysis of iNOS protein and NO production by Western blotting and Griess reagent, respectively (Fig. 4b). Similarly, GST–NSP4 (from 100 to 1 pmol) markedly induced expression of iNOS protein and NO production in RAW264.7 cells in a dose-dependent manner (Fig. 4b, lanes 3–5). No effect was observed in GST- or GST–VP8-treated cells (Fig. 4b, lanes 7 and 8). GST–NSP4s from other avian rotaviruses, turkey strains Ty-1 and Ty-3 and chicken strain Ch-1 were also able to induce iNOS expression and NO production in RAW264.7 cells, indicating the universality of the stimulatory effect of NSP4 on macrophages, at least among avian rotaviruses (data not shown). To confirm further the specificity of NSP4 inducibility, GST–NSP4 was treated with normal or polyclonal anti-PO-13 NSP4 guinea pig serum for 1 h at 37 °C prior to stimulation of RAW264.7 cells. The results showed that anti-NSP4 serum, but not normal serum, significantly reduced the GST–NSP4 inducibility of NO production and iNOS mRNA expression in RAW264.7 cells (Fisher's test, $P<0.02$) (Fig. 5).

Mapping of the domain of NSP4 responsible for iNOS expression and NO production

To determine the functional domain of NSP4 responsible for iNOS induction and NO production in macrophages, RAW264.7 cells were treated with tenfold serial dilutions (from 200 to 0.2 pmol) of GST-truncated PO-13 NSP4 fusion proteins (Fig. 6a). Macrophages treated with GST only exhibited low levels of iNOS protein expression and NO production (Fig. 6b and c, lane 1). This may have been

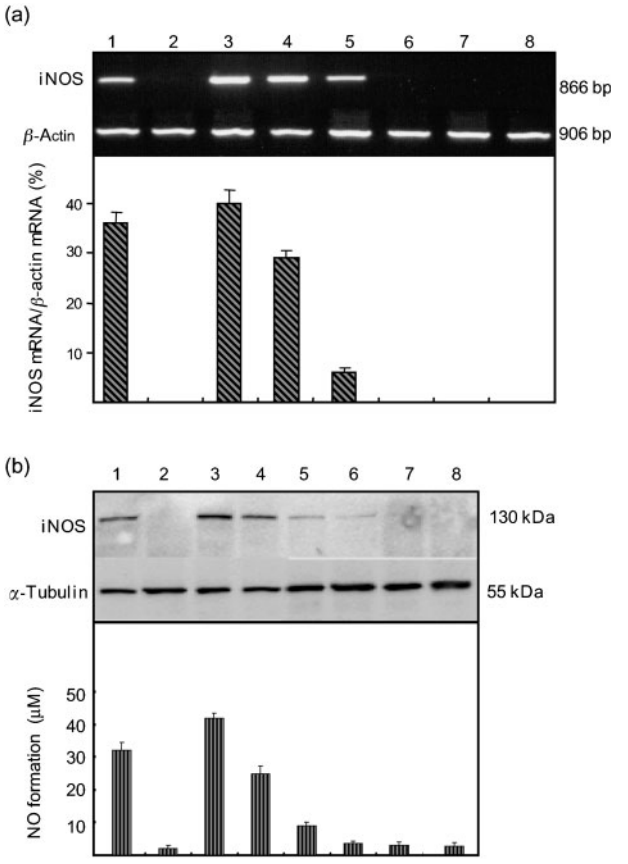

Fig. 4. Dose-dependent increases in iNOS mRNA levels in peritoneal macrophages and iNOS protein levels and NO production in RAW264.7 cells after treatment with NSP4. (a) Peritoneal macrophages collected from adult CD-1 mice were plated in a six-well plate (5×10^6 cells per well) and stimulated for 4 h with LPS (5 μg, lane 1) or left untreated (lane 2), tenfold serial dilutions of GST–NSP4 (from 100 to 0.1 pmol, lanes 3–6), GST (200 pmol, lane 7) or GST–VP8 (200 pmol, lane 8). The mRNA levels of iNOS were determined by RT-PCR using specific primers. The upper panel shows representative results of RT-PCR analysis of iNOS mRNA (866 bp) and control β-actin mRNA (906 bp). The lower panel shows the ratios of iNOS mRNA and β-actin mRNA from the relative intensities analysed by NIH IMAGE 1.62 software. Values are means ± SD derived from three independent experiments. (b) RAW264.7 cells were plated in a 24-well plate (10^6 cells per well), incubated for 24 h at 37 °C and then stimulated for 24 h with LPS (5 μg, lane 1) or left untreated (lane 2), tenfold serial dilutions of GST–NSP4 (from 100 to 0.1 pmol, lanes 3–6), GST (200 pmol, lane 7) or GST–VP8 (200 pmol, lane 8). The upper panel shows representative results of Western blotting analysis of iNOS protein (130 kDa) and control α-tubulin (55 kDa). Twenty-five micrograms of total protein were loaded into each lane. The blot was probed with a polyclonal antibody specific to mouse iNOS peptide or with a monoclonal antibody specific to murine α-tubulin. The lower panel shows NO production in the culture medium of RAW264.7 cells treated as described above for 24 h as measured by Griess reaction. Values in the lower panel are means ± SD derived from three independent experiments.

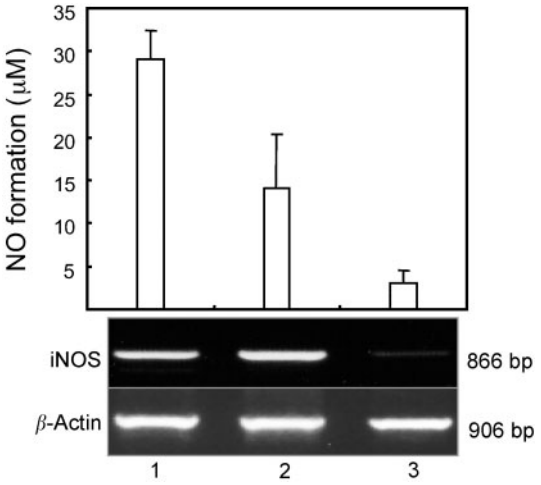

Fig. 5. Inhibition of NSP4 inducibility of iNOS mRNA expression and NO production by anti-NSP4 polyclonal serum. RAW264.7 cells were plated in a six-well plate (10^6 cells per well), incubated for 24 h at 37 °C and then stimulated with LPS (5 μg, lane 1), GST–NSP4 pre-treated with normal guinea pig serum (10 pmol, lane 2) or pre-treated with anti-PO-13 NSP4 guinea pig serum (10 pmol, lane 3). The upper panel shows NO production in the culture media after a 12 h incubation as determined by Griess reaction. The data are means ± SD of triplicate experiments. The lower panel shows iNOS mRNA induction after a 4 h incubation as determined by RT-PCR.

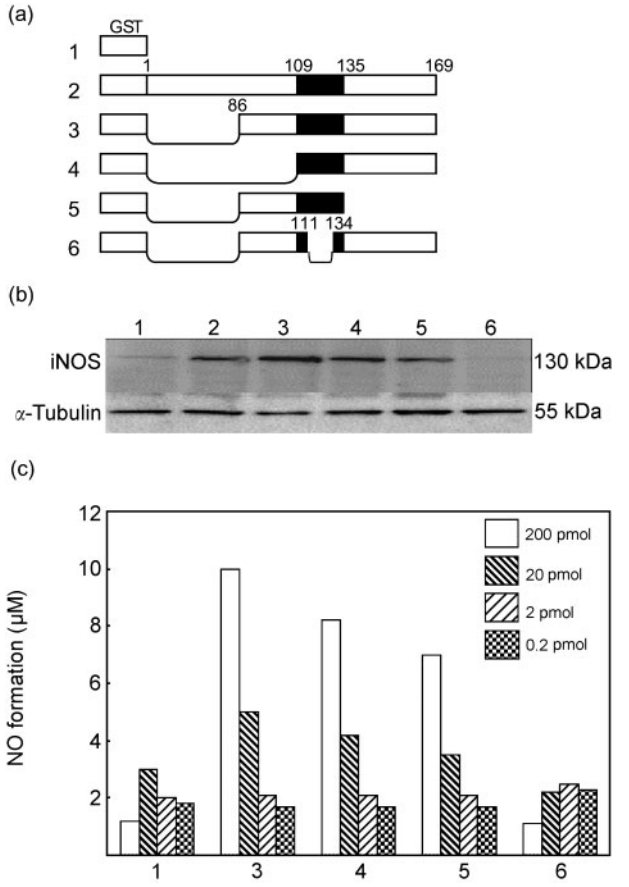

Fig. 6. Induction of iNOS protein synthesis and NO production by truncated NSP4s. RAW264.7 cells were plated in a 24-well plate (10^6 cells per well), incubated for 24 h at 37 °C and then stimulated for 24 h with tenfold serial dilutions of GST or truncated GST–NSP4s (from 200 to 0.2 pmol). (a) Schematic representation of GST–NSP4, the four GST-truncated NSP4s and GST. Shaded boxes represent GST and filled boxes represent the region corresponding to the enterotoxin domain (aa 109–135) of PO-13 NSP4 (Mori *et al.*, 2002; Borgan *et al.*, 2003). (b) Representative results of Western blots of iNOS protein (130 kDa) and control α-tubulin (55 kDa) induced by 200 pmol truncated GST–NSP4s. One hundred micrograms of total protein was loaded into each lane. The blot was probed with a polyclonal antibody specific to mouse iNOS peptide or with a monoclonal antibody specific to murine α-tubulin. (c) NO production in the culture medium of RAW264.7 cells treated as described above for 24 h as measured by Griess reaction. 1, GST; 2, GST–NSP4; 3, GST–$NSP4_{86–169}$; 4, GST–$NSP4_{109–169}$; 5, GST–$NSP4_{86–135}$; 6, GST–$NSP4_{86–169Δ112–133}$.

mainly due to a low level of constitutive iNOS expression, as suggested by some reports (Hoffman *et al.*, 1997). GST–$NSP4_{86–169}$, GST–$NSP4_{109–169}$ and GST–$NSP4_{86–135}$ have been shown to be biologically active and to be able to induce diarrhoea in suckling mice (Mori *et al.*, 2002). GST–$NSP4_{86–169}$, GST–$NSP4_{109–169}$ and GST–$NSP4_{86–135}$ induced expression of iNOS protein and NO production in RAW264.7 cells (Fig. 6b and c, lanes 3–5). Although induction with the truncated proteins was slightly weaker than with GST–NSP4, these results were significantly different by Fisher's direct test ($P<0.01$) from the results obtained by GST treatment (Fig. 6b and c, lane 1). These proteins overlapped each other over aa 109–135. In contrast, no induction was observed in RAW264.7 cells treated with GST–$NSP4_{86–169Δ112–133}$, in which aa 112–133 were deleted from GST–$NSP4_{86–169}$ (Fig. 6b and c, lane 6), indicating that the region aa 109–135 of PO-13 NSP4 has inductive activity for iNOS in macrophages.

DISCUSSION

In order to understand the host response to rotavirus infection in more detail, we investigated the transcriptional changes of iNOS mRNA in the murine ileum (Fig. 1). It is known that rotavirus infection can selectively induce chemokines and cytokines in intestinal epithelial cells (Rollo *et al.*, 1999). We showed that expression of ileal iNOS mRNA is upregulated during diarrhoea caused in suckling mice by avian, simian and murine rotaviruses. The time course of ileal iNOS induction showed that expression of iNOS mRNA was rapidly upregulated within 6 h p.i. and remained elevated up to 36 h p.i. Although iNOS was induced before the onset of clinical diarrhoea, iNOS elevation paralleled the occurrence of diarrhoea. Decline of

iNOS mRNA expression while diarrhoea peaked may call into question the potential role of NO in rotavirus diarrhoea. However, expression of iNOS is known to be regulated primarily by the *de novo* synthesis and stability of iNOS mRNA and protein (MacMicking *et al.*, 1997; Rodriguez-Pascual *et al.*, 2000; Carpenter *et al.*, 2001). Furthermore, the diarrhoeal response seen after the decline of iNOS mRNA expression may be also explained by the changes in intestinal homeostasis and morphology caused by virus replication (Boshuizen *et al.*, 2003).

Peaks of ileal iNOS mRNA expression were observed at 12 and 36 h p.i. The occurrence of two peaks during rotavirus infection is in agreement with results of a recent study showing two peaks of rotavirus replication in small-intestinal tissue at 1 and 4 days p.i. (Boshuizen *et al.*, 2003). The differences between peaks observed in our study (at 12 and 36 h p.i) and those observed in a previous study (1 and 4 days p.i.) may be due to differences in the parameters measured (iNOS vs virus replication), as well as to mouse strains, rotavirus strains and the initial rotavirus load used to induce diarrhoea (10^7 f.f.u. vs 2×10^4 f.f.u.). Our results also suggest the dependency of iNOS mRNA expression on rotavirus replication, as shown by the inability of PI-SA11 to induce iNOS expression (Fig. 3a). This suggests that the accumulation of viral products is important for induction of ileal iNOS expression. As NSP4 only induced iNOS expression in the murine ileum, it is thought that NSP4 is at least one among other effectors (host and/or viral) that directly induce iNOS expression during rotavirus infection *in vivo*. In this respect, it is now known that dsRNA can also induce NO through a toll-like receptor 3-dependent pathway (Alexopoulou *et al.*, 2001). Therefore, the possibility of roles of rotavirus dsRNA, other viral proteins and rotavirus-induced cytokines in the induction of ileal iNOS mRNA should not be excluded. Unfortunately, the lack of a reverse genetics system for rotavirus and the current impracticability of some available techniques such as RNA interference largely preclude the assessment of the potential roles of individual viral proteins in many steps of the pathogenicity of rotavirus.

The results of this study showed that NSP4 strongly and specifically induces iNOS expression and NO production in murine peritoneal macrophages and RAW264.7 cells, as well as in the murine ileum (Fig. 4). As a major part of innate immunity, iNOS is induced in macrophages in numerous diseases caused by various pathogens (MacMicking *et al.*, 1997). Most bacteria induce iNOS expression in macrophages after invasion (Bekker *et al.*, 2001) or through an LPS-mediated pathway (Xie *et al.*, 1992). In contrast to the action of LPS on macrophages, NSP4 was able to induce significant iNOS expression, even at a concentration as low as 1 pmol, suggesting either that NSP4 is a potent stimulator for macrophages or that simultaneous NSP4-mediated events potentiate NSP4 action. It has recently been reported that intracellular calcium may contribute to iNOS induction in murine macrophages (Chen *et al.*, 1998). Therefore, the potential role of NSP4-elicited intracellular calcium elevation in iNOS expression remains to be determined. On the other hand, the ability of NSP4 at a minute concentration (as low as 1 pmol) to induce iNOS expression in macrophages suggests that high-affinity NSP4 receptors are present on macrophages, although this remains to be proven.

NSP4 was also able to induce iNOS mRNA expression within 4 h in the murine ileum when the ileum was treated with NSP4 *ex vivo*. As the whole ileum was used to assess iNOS induction by NSP4, it is not clear whether the source of iNOS was intestinal epithelial cells or resident monocytes/macrophages. In fact, iNOS has been induced under various conditions in many intestinal cell lines such as the human adenocarcinoma cell lines HT-29/cl.19A and Caco-2 (Salzman *et al.*, 1998; Resta-Lenert & Barrett, 2002) and in T84 colon epithelial cells (Korhonen *et al.*, 2001). We tried to induce iNOS expression by NSP4 in Caco-2 cells, but no stimulation was observed (data not shown). However, the possibility of the role of intestinal epithelial cells cannot be excluded, as some *in vitro* models may not reconstitute all of the biochemical and molecular responses of the native epithelium. Furthermore, important species differences between the promoter regions of human and murine *inos* have been reported to be of functional significance (Nathan & Xie, 1994; Vallance & Charles, 1998). Whereas murine iNOS expression is rapidly induced upon exposure to certain stimulators, it has often proved difficult to induce functionally active iNOS in human cells and tissues *in vitro* (Vallance & Charles, 1998).

Many studies have demonstrated that rotavirus NSP4s from various animal and avian species have enterotoxigenic activities in suckling mice (Ball *et al.*, 1996; Morris *et al.*, 1999; Mori *et al.*, 2002). Enterotoxigenic activity of NSP4 has been attributed to aa 109–135 (Ball *et al.*, 1996; Mori *et al.*, 2002). Our results showed that the inductive domain of iNOS on NSP4 exists within the enterotoxin domain, as demonstrated by experiments using a series of truncated NSP4s (Fig. 6). This suggests that NSP4 may exert its toxic activity by inducing NO production. Indeed, the enterotoxin domain of NSP4 is an active region with multifunctional activities such as intracellular Ca^{2+} mobilization, lipid membrane binding and Na^{+}–D-glucose symporter inhibition (Dong *et al.*, 1997; Halaihel *et al.*, 2000; Huang *et al.*, 2001). It is notable, however, that truncated NSP4s are less active than the full protein in inducing NO production, as larger amounts of truncated NSP4s were necessary for induction compared with the complete NSP4 (Fig. 6c). This finding has also been reported for NSP4 in inducing diarrhoea (Ball *et al.*, 1996; Mori *et al.*, 2002), suggesting that NSP4 needs to fold into its correct conformation for full functional activity.

Rodriguez-Diaz *et al.* (2006) have also investigated the role of NO during clinical and experimental rotavirus infection. They showed that iNOS mRNA is upregulated in the ileum upon rotavirus infection in mice. They also reported that NSP4 can inactivate Ca^{2+}-dependent constitutive NOS, resulting in NO production within minutes in HT-29 cells.

This observation is interesting, as we were not able to induce NO production in Caco-2 cells. This discrepancy might be explained in part by the fact that different cell lines respond to rotavirus and/or NSP4 in different ways. Holloway & Coulson (2006) have recently shown that rotavirus infection can activate p38 in Caco-2 and MA104 cells but not in HT-29 cells, suggesting that rotavirus activates distinct pathways in different cell lines. Moreover, Rodriguez-Diaz *et al.* (2006) did not show whether induction of Ca^{2+}-dependent constitutive NOS was universal among different cell lines.

The association of iNOS induction with diarrhoea has been suggested for other infectious diseases such as *Shigella* and *Cholera* infections (Turvill *et al.*, 1999; Rhee *et al.*, 2001; Fasano, 2002). Elevation of iNOS mRNA before the onset of clinical diarrhoea suggests a role for NO in initiation of the diarrhoeal response. NO may later only sustain the diarrhoeal response, as suggested by the decline of iNOS mRNA expression while diarrhoea peaked. Indeed, three observations suggest that NO acts as a potentiator or initiator of rotavirus-induced diarrhoea. First, we found in this study that iNOS expression is elevated in response to reproductive rotavirus infection and that this elevation correlated with the occurrence of diarrhoea. Secondly, we previously showed that the enterotoxin NSP4, which is known to induce diarrhoea in neonatal mice within 2–4 h, was able to induce iNOS when it was administrated on its own to the murine ileum and that this induction occurred within 4 h. Thirdly, the fact that the enterotoxin domain of NSP4 (aa 109–135) is involved in NSP4 inducibility of iNOS may provide evidence that elevation of iNOS expression is associated with rotavirus diarrhoea. Future experimental approaches such as the use of L-arginine analogues, the use of specific iNOS inhibitors or the evaluation of rotavirus infectivity in iNOS-knockout mice are needed to address the role of NO in rotavirus pathogenicity.

ACKNOWLEDGEMENTS

We thank Dr H. B. Greenberg, Stanford University, USA, for providing the murine rotavirus EW. We also thank Dr O. Nakagomi, Akita University, for providing the simian rotavirus SA-11. This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sport, Science and Technology, Japan (nos 13460141 and 15580273), and a Grant-in-Aid for scientific research [The 21st Century Center-of-Excellence Program from the Ministry of Education, Culture, Sports, Science and Technology, Japan (E-1)].

REFERENCES

Alexopoulou, L., Holt, A. C., Medzhitov, R. & Flavell, R. A. (2001). Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. *Nature* **413**, 732–738.

Ball, J. M., Tian, P., Zeng, C. Q.-Y., Morris, A. P. & Estes, M. K. (1996). Age-dependent diarrhea induced by a rotaviral nonstructural glycoprotein. *Science* **272**, 101–104.

Bekker, L. G., Freeman, S., Murray, P. J., Ryffel, B. & Kaplan, G. (2001). TNF-α controls intracellular mycobacterial growth by inducible nitric oxide synthase-dependent and inducible nitric oxide synthase-independent pathways. *J Immunol* **166**, 6728–6734.

Borgan, M. A., Mori, Y., Ito, N., Sugiyama, M. & Minamoto, N. (2003). Antigenic analysis of nonstructural protein (NSP) 4 of avian rotavirus PO-13 strain. *Microbiol Immunol* **47**, 661–668.

Boshuizen, J. A., Reimerink, J. H. J., Male, A. M., Van Ham, V. J. J., Koopmans, M. P. G., Büller, H. A., Dekker, J. & Einerhand, A. W. C. (2003). Changes in small intestinal homeostasis, morphology, and gene expression during rotavirus infection of infant mice. *J Virol* **77**, 13005–13016.

Bult, H., Boeckxstaens, G. E., Pelckmans, P. A., Jordaens, F. H., Van Maercke, Y. M. & Herman, A. G. (1990). Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. *Nature* **345**, 346–347.

Carpenter, L., Cordery, D. & Biden, T. J. (2001). Protein kinase Cδ activation by interleukin-1β stabilizes inducible nitric oxide synthase mRNA in pancreatic β-cells. *J Biol Chem* **276**, 5368–5374.

Chen, B. C., Chou, C. & Lin, W. (1998). Pyrimidinoceptor-mediated potentiation of inducible nitric-oxide synthase induction in J774 macrophages. *J Biol Chem* **273**, 29754–29763.

Davidson, G. P. & Barnes, G. L. (1979). Structural and functional abnormalities of the small intestine in infants and young children with rotavirus enteritis. *Acta Paediatr Scand* **68**, 181–186.

Dickman, K. G., Hempson, S. J., Anderson, J., Lippe, S., Zhao, L., Burakoff, R. & Shaw, R. D. (2000). Rotavirus alters paracellular permeability and energy metabolism in Caco-2 cells. *Am J Physiol Gastrointest Liver Physiol* **279**, G757–G766.

Dong, Y., Zeng, C. Q.-Y., Ball, J. M., Estes, M. K. & Morris, A. P. (1997). The rotavirus enterotoxin NSP4 mobilizes intracellular calcium in human intestinal cells by stimulating phospholipase C-mediated inositol 1,4,5-trisphosphate production. *Proc Natl Acad Sci U S A* **94**, 3960–3965.

Fasano, A. (2002). Toxins and the gut: role in human disease. *Gut* **50** (Suppl. III), iii9–iii14.

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S. & Tannenbaum, S. R. (1982). Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. *Anal Biochem* **126**, 131–138.

Greenberg, H. B., Clark, H. F. & Offit, P. A. (1994). Rotavirus pathology and pathophysiology. *Curr Top Microbiol Immunol* **185**, 255–283.

Groene, W. S. & Shaw, R. D. (1992). Psoralen preparation of antigenically intact noninfectious rotavirus particles. *J Virol Methods* **38**, 93–102.

Halaihel, N., Lievin, V., Ball, J. M., Estes, M. K., Alvarado, F. & Vasseur, M. (2000). Direct inhibitory effect of rotavirus NSP4(114–135) peptide on the Na^+-D-glucose symporter of rabbit intestinal brush border membrane. *J Virol* **74**, 9464–9470.

Hoffman, R. A., Zhang, G., Nüssler, N. C., Gleixner, S. L., Ford, H. R., Simmons, R. L. & Watkins, S. C. (1997). Constitutive expression of inducible nitric oxide synthase in the mouse ileal mucosa. *Am J Physiol Gastrointest Liver Physiol* **272**, G383–G392.

Holloway, G. & Coulson, B. S. (2006). Rotavirus activates JNK and p38 signaling pathways in intestinal cells, leading to AP-1-driven transcriptional responses and enhanced virus replication. *J Virol* **80**, 10624–10633.

Huang, H., Schroeder, F., Zeng, C., Estes, M. K., Schoer, J. K. & Ball, J. M. (2001). Membrane interactions of a novel viral enterotoxin: rotavirus nonstructural glycoprotein NSP4. *Biochemistry* **40**, 4169–4180.

Izzo, A. A., Mascolo, N. & Capasso, F. (1998). Nitric oxide as a modulator of intestinal water and electrolyte transport. *Dig Dis Sci* **43**, 1605–1620.

Jourdan, N., Brunet, J. P., Sapin, C., Blais, A., Cotte-Laffitte, J., Forestier, F., Quero, A. M., Trugnan, G. & Servin, L. A. (1998). Rotavirus infection reduces sucrase-isomaltase expression in human intestinal epithelial cells by perturbing protein targeting and organization of microvillar cytoskeleton. *J Virol* **72**, 7228–7236.

Jung, H. C., Eckmann, L., Yang, S. K., Panja, A., Fierer, J., Morzycka-Wroblewska, E. & Kagnoff, M. F. (1995). A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. *J Clin Invest* **95**, 55–65.

Kapikian, A. Z. & Chanock, R. M. (1990). Rotaviruses. In *Virology*, 2nd edn, pp. 1353–1404. Edited by B. N. Fields, D. M. Knipe, M. S. Hirsch, L. M. Joseph, T. P. Monath & B. Roizman. New York: Raven.

Korhonen, R., Korpela, R., Saxelin, M., Mäki, M., Kankaanranta, H. & Moilanen, E. (2001). Induction of nitric oxide synthesis by probiotic *Lactobacillus rhamnosus* GG in J774 macrophages and human T84 intestinal epithelial cells. *Inflammation* **25**, 223–232.

Kubes, P. (1992). Nitric oxide modulates epithelial permeability in the feline small intestine. *Am J Physiol* **262**, G1138–G1142.

Londrigan, S. L., Graham, K. L., Takada, Y., Halasz, P. & Coulson, B. S. (2003). Monkey rotavirus binding to $\alpha 2\beta 1$ integrin requires the $\alpha 2$ I domain and is facilitated by the homologous $\beta 1$ subunit. *J Virol* **77**, 9486–9501.

Lundgren, O., Peregrin, A. T., Persson, K., Kordasti, S., Uhnoo, I. & Svensson, L. (2000). Role of the enteric nervous system in the fluid and electrolytes secretion of rotavirus diarrhea. *Science* **287**, 491–495.

MacMicking, J., Xie, Q. W. & Nathan, C. (1997). Nitric oxide and macrophage function. *Annu Rev Immunol* **15**, 323–350.

MacNaughton, W. K. (1993). Nitric oxide-donating compounds stimulate electrolyte transport in the guinea pig intestine *in vitro*. *Life Sci* **53**, 585–593.

McNeal, M. M., Rae, M. N., Bean, J. A. & Ward, R. L. (1999). Antibody-dependent and -independent protection following intranasal immunization of mice with rotavirus particles. *J Virol* **73**, 7565–7573.

Minamoto, N., Oki, K., Tomita, M., Kinjo, T. & Suzuki, Y. (1988). Isolation and characterization of rotavirus from feral pigeon in mammalian cell cultures. *Epidemiol Infect* **100**, 481–492.

Mori, Y., Sugiyama, M., Takayama, M., Atoji, Y., Masegi, T. & Minamoto, N. (2001). Avian-to-mammal transmission of an avian rotavirus: analysis of its pathogenicity in a heterologous mouse model. *Virology* **288**, 63–70.

Mori, Y., Borgan, M. A., Ito, N., Sugiyama, M. & Minamoto, N. (2002). Diarrhea-inducing activity in suckling mice of avian rotavirus NSP4 glycoproteins, which differ greatly from mammalian rotavirus NSP4 glycoproteins in deduced amino acid sequences. *J Virol* **76**, 5829–5834.

Morris, A. P., Scott, J. K., Ball, J. M., Zeng, C. Q., O'Neal, W. K. & Estes, M. K. (1999). NSP4 elicits age-dependent diarrhea and Ca^{2+}-mediated I^- influx into intestinal crypts of CF mice. *Am J Physiol* **277**, G431–G444.

Nathan, C. & Xie, Q.-W. (1994). Nitric oxide synthases: roles, tolls, and controls. *Cell* **78**, 915–918.

Osborne, M. P., Haddon, S. J., Worton, K. J., Spencer, A. J., Starkey, W. G., Thornber, D. & Stephen, J. (1991). Rotavirus-induced changes in microcirculation of intestinal villi of neonatal mice in relation to the induction and persistence of diarrhea. *J Pediatr Gastroenterol Nutr* **12**, 111–120.

Perrin, P., Tino De Franco, M., Jallet, C., Fouque, F., Morgeaux, S., Tordo, N. & Colle, J. H. (1996). The antigen-specific cell-mediated immune response in mice is suppressed by infection with pathogenic lyssaviruses. *Res Virol* **147**, 289–299.

Rhee, S. J., Wilson, K. T., Gobert, A. P., Nataro, J. P. & Fasano, A. (2001). The enterotoxic activity of *Shigella* enterotoxin 1 (ShET1) is mediated by inducible nitric oxide synthase activity. *J Pediatr Gastroenterol Nutr* **33**, 400–416.

Resta-Lenert, S. & Barrett, K. E. (2002). Enteroinvasive bacteria alter barrier and transport properties of human intestinal epithelium: role of iNOS and COX-2. *Gastroenterology* **122**, 1070–1087.

Rodriguez-Diaz, J., Banasaz, M., Istrate, C., Buesa, J., Lundgren, O., Espinoza, F., Sundqvist, T., Rottenberg, M. & Svensson, L. (2006). Role of nitric oxide during rotavirus infection. *J Med Virol* **78**, 979–985.

Rodriguez-Pascual, F., Hausding, M., Ihrig-Biedert, I., Furneaux, H., Levy, A. P., Forstermann, U. & Kleinert, H. (2000). Complex contribution of the 3′-untranslated region to the expressional regulation of the human inducible nitric-oxide synthase gene. Involvement of the RNA-binding protein HuR. *J Biol Chem* **275**, 26040–26049.

Rolfe, V. E. & Milla, P. J. (1999). Nitric oxide stimulates cyclic guanosine monophosphate production and electrogenic secretion in Caco-2 colonocytes. *Clin Sci* **96**, 165–170.

Rollo, E. E., Kumar, K. P., Reich, N. C., Cohen, J., Angel, J., Greenberg, H. B., Sheth, R., Anderson, J., Oh, B. & other authors (1999). The epithelial cell response to rotavirus infection. *J Immunol* **163**, 4442–4452.

Salzman, A. L., Eaves-Pyles, T., Linn, S. C., Denenberg, A. G. & Szabó, C. (1998). Bacterial induction of inducible nitric oxide synthase in cultured human intestinal epithelial cells. *Gastroenterology* **114**, 93–102.

Stack, W. A., Filipowicz, B. & Hawkey, C. J. (1996). Nitric oxide donating compounds stimulate human colonic ion transport *in vitro*. *Gut* **39**, 93–99.

Turvill, J. L., Mourad, F. H. & Farthing, M. J. (1999). Proabsorptive and prosecretory roles for nitric oxide in cholera toxin induced secretion. *Gut* **44**, 33–39.

Vallance, P. & Charles, I. (1998). Nitric oxide in sepsis: of mice and men. *Sepsis* **1**, 93–100.

Wilson, K. T., Xie, Y., Musch, M. W. & Chang, E. B. (1993). Sodium nitroprusside stimulates anion secretion and inhibits NaCl absorption in rat colon. *J Pharmacol Exp Ther* **266**, 224–230.

Xie, Q. W., Cho, H. J., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., Ding, A., Troso, T. & Nathan, C. (1992). Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* **256**, 225–227.