Inhibition of rotavirus ECwt infection in ICR suckling mice by N-acetylcysteine, peroxisome proliferator-activated receptor gamma agonists and cyclooxygenase-2 inhibitors

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Live attenuated vaccines have recently been introduced for preventing rotavirus disease in children. However, alternative strategies for prevention and treatment of rotavirus infection are needed mainly in developing countries where low vaccine coverage occurs. In the present work, N-acetylcysteine (NAC), ascorbic acid (AA), some nonsteroidal anti-inflammatory drugs (NSAIDs) and peroxisome proliferator-activated receptor gamma (PPARγ) agonists were tested for their ability to interfere with rotavirus ECwt infectivity as detected by the percentage of viral antigen-positive cells of small intestinal villi isolated from ECwt-infected ICR mice. Administration of 6 mg NAC/kg every 8 h for three days following the first diarrhoeal episode reduced viral infectivity by about 90%. Administration of AA, ibuprofen, diclofenac, pioglitazone or rosiglitazone decreased viral infectivity by about 55%, 90%, 35%, 32% and 25%, respectively. ECwt infection of mice increased expression of cyclooxygenase-2, ERp57, Hsc70, NF-κB, Hsp70, protein disulphide isomerase (PDI) and PPARγ in intestinal villus cells. NAC treatment of ECwt-infected mice reduced Hsc70 and PDI expression to levels similar to those observed in villi from uninfected control mice. The present results suggest that the drugs tested in the present work could be assayed in preventing or treating rotaviral diarrhoea in children and young animals.

Key words: rotavirus infection - N-acetylcysteine - thiazolidinediones - NSAIDs - ICR mice

Rotaviruses belong to the family Reoviridae and are the major etiological agents of severe diarrhoea affecting children less than five years of age worldwide (Parashar et al. 2006, Esposito et al. 2011). Their virions are composed of three concentric protein layers that encapsidate 11 double-stranded RNA genome segments (Estes & Kapikian 2007). Over 500,000 children, most of them from developing countries, die annually from rotavirus infection and many more require hospitalisation for treatment of the severe and dehydrating rotavirus-associated diarrhoea (Parashar et al. 2006, Danchin & Bines 2009). An important reduction in rotavirus-caused deaths has been reported as a consequence of the use of two recently introduced rotavirus vaccines (RotaTeq, Merck, and Rotarix, GlaxoSmithKline Biologicals) (Nelson & Glass 2010, Santosham 2010). However, the potential of rotavirus vaccines for reducing the risk of death from diarrhoea in the poorest countries has been challenged by logistical problems, the age-restricted recommendation for vaccine administration, the low vaccine coverage and the low on-time immunisation (Clark & Sanderson 2009, Santosham 2010). The above facts justify the attempts aimed at developing alternative or complementary strategies for preventing or treating the rotavirus-associated diarrhoea.

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Balancing reduction and oxidation seems to be a crucial event for maintaining life while aging has been considered a process involving gradual oxidation (Kregel & Zhang 2007, Cui et al. 2012). Cellular mechanisms that deal with damaging oxidative environments include redox systems such as glutathione (GSH)/GSSG (the GHS system), NADH/NAD^+, NADPH/$NADP^+$ and Trx(SH)2/Trx(S-S) (the thioredoxin system). Oxidative stress has been shown to be involved in the pathogenesis of viral infections by human immunodeficiency virus (HIV), influenza virus and dengue virus in which GSH levels have been found to be decreased and reactive oxygen species (ROS) levels increased (Garland & Fawzi 1999, Cai et al. 2003, Nencioni et al. 2003, Tian et al. 2010). Redox imbalance has also been reported to occur in infections caused by rabies virus (Jackson et al. 2010), herpes simplex virus type 1 (Kavouras et al. 2007), hepatitis C virus (Clément et al. 2009) and hepatitis B virus (Severi et al. 2006). N-acetylcysteine (NAC), an anti-oxidant that can function in the body as precursor of GSH (Atkuri et al. 2007), has been used in the treatment of influenza virus infections (de Flora et al. 1997, Ghezzi & Ungheri 2004, Garozzo et al. 2007, Lai et al. 2010) and also in the treatment of dengue-associated fulminant liver failure (Lim & Lee 2012). It has been found that I-152, a pro-drug of NAC and GSH, was able to inhibit murine acquired immune deficiency syndrome (Ho & Dougla 1992, Fraternale et al. 2008). GSH and NAC have been shown to inhibit the induction of HIV-1 expression in human monocyte/macrophages (Ho & Dougla 1992).

Traditional nonsteroidal anti-inflammatory drugs (NSAIDs) are known to inhibit both isoforms of cycloox-

ygenase (COX)-1/2, key enzymes in the production of prostaglandins (Rao & Knaus 2008). Induction of COX-2 expression appears to be regulated by extracellular signal-regulated protein kinases (ERK)1/2 and p38 mitogen-activated protein kinase (MAPK) pathways and transcription factors, such as nuclear factor kappa B (NF-κB) (Newton et al. 1997, Bartlett et al. 1999, Subbaramaiah et al. 2000, Charalambous et al. 2003). PKA-mediated ERK1/2, p38 MAPK and NF-κB pathways have been shown to be involved in the COX-2 activity induction during rotavirus infection (Rossen et al. 2004). NF-κB activation has been shown to occur during infection by several viruses, including rotaviruses (Roulston et al. 1999, Rossen et al. 2004). Rotaviruses have also been found to activate NF-κB-dependent gene expression and induce the increased expression of interleukin-8 and other cytokines (Sheth et al. 1996, Rollo et al. 1999, LaMonica et al. 2001, Casola et al. 2002). It has been shown that indomethacin (a COX-1 and COX-2 inhibitor) treatment of human intestinal Caco-2 cells significantly reduced rotavirus infectivity by affecting a post-binding step and inhibiting virus-directed protein synthesis (Rossen et al. 2004). On the other hand, peroxisome proliferator-activated receptor gamma (PPARγ) ligands have been found to down-regulate the transcriptional activation of COX-2 through multiple mechanisms (Subbaramaiah et al. 2001), which include the inhibition of some NF-κB pathway steps (Straus et al. 2000). PPARγ modulates ROS generation and activation of redox-sensitive NF-κB and hypoxia-inducible factor 1-alpha in mice (Lee et al. 2006). Central inflammatory pathways such as NF-κB, activator protein 1 and STAT have been found to be antagonised by PPARs in monocyte-macrophages, whereas down-regulation of these signalling pathways by thiazolidine-2-4-diones (TZDs), including pioglitazone (PGZ) and rosiglitazone (RGZ), has been shown to lead to reduced levels of oxidative products (Jiang et al. 1998). It has been found that TZDs were able to attenuate hyperglycaemia-induced ROS production by inducing manganese superoxide dismutase and promoting mitochondrial biogenesis (Fujisawa et al. 2009). Recently, PPARγ agonists have been suggested as a potential approach for down-regulating the inflammatory response to virus-induced inflammation (Bassaganya-Riera et al. 2010).

Although rotavirus diarrhoea has been attributed to different mechanisms, rotaviruses proliferation in the non-dividing mature enterocytes localised near the tips of the villi is a factor contributing to induce diarrhoea (Ramig 2004). Homeostatic control of the intestinal redox environment seems to be a critical factor for maintaining intestinal functions. Cells from intestinal epithelium are exposed to oxidative damage induced by oxidant agents present in the luminal environment. Mucosal integrity is ensured by the luminal redox balance of the GSH/GSSG and cysteine/cystine (Cys/CySS) couples, which are also involved in supporting luminal nutrient absorption, mucus fluidity and microbiota (Go & Jones 2008, Jones & Go 2010, Circu & Aw 2012). Normal intestinal cell transition from proliferative state to non-dividing differentiated state or apoptosis has been associated with increasing oxidation of intracellular GSH/GSSG or extracellular Cys/CySS redox systems (Jones 2006, Jones & Go 2010). Recent advances in intestinal redox biology suggest that the loss of intestinal homeostasis caused by oxidative stress in the mucosal and adjacent tissues can alter nutrient digestion and absorption, stem cell proliferation, enterocyte apoptosis and immune response (Circu & Aw 2012).

Rotavirus infectivity has recently been shown to be inhibited by the treatment of cells with membrane impermeant thiol/disulfide exchange inhibitors and antibodies (Abs) against protein disulphide isomerase (PDI), which suggested that infectivity was dependent on cell surface oxidation-reduction reactions (Calderon et al. 2012). Some findings have suggested that the rotavirus structural protein (SP) VP7 is a substrate for the endoplasmic reticulum (ER)-associated oxidant PDI (Svensson et al. 1994, Mirazimi & Svensson 1998, Maruri-Avidal et al. 2008). In addition, NAC, NASIDs and PPARγ agonists have recently been found to inhibit rotavirus infection in cultured MA104 cells (Guerrero et al. 2012). Understanding the molecular and cellular disorders caused in vivo by rotavirus infection, such as oxidative stress, could be useful in designing therapeutic strategies aimed at interfering with the cell injury associated with the rotavirus-induced pro-oxidant status. The present work provides evidence that rotavirus infectivity is inhibited in ICR mice treated with NAC, PGZ or RGZ, which are drugs that down-regulate the NF-κB pathway involved in the transcriptional activation of COX-2 reported to occur during rotavirus infection (Rossen et al. 2004).

MATERIALS AND METHODS

Virus, animals and drugs - Rotavirus ECwt (wild-type murine rotavirus EDIM-Cambridge) was kindly provided by Dr M Franco (Genetics Institute, Pontifical Xaverian University, Bogotá, Colombia) and propagated by orally inoculating suckling ICR mice (10-12 days old) with cesium chloride-purified virus preparations as previously described (Guerrero et al. 2010). ICR mice were obtained from the National Institute of Health (Bogotá, Colombia). After cervical dislocation, the small intestines were removed from mice for villus isolation or virus purification. The present study was approved by the Ethical Committee of the School of Medicine, National University of Colombia, and performed according to the established guidelines. Drugs used were according to the United States Pharmacopeia grade and consisted of active ingredients lacking excipients. NSAIDs [diclofenac (DCF) and ibuprofen (IBF)] and antioxidants [NAC and ascorbic acid (AA)] were purchased from MP Biomedicals (Solon, OH, USA). PPARγ agonists, PGZ and RGZ, were provided by Sigma (St. Louis, MO, USA) and Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA), respectively. Nitazoxanide (NTZ) was obtained from Santa Cruz Biotechnology Inc. Drugs were solubilised in the solvent indicated by manufacturers and then diluted in sterile phosphate buffered saline (PBS) and sterilised by filtration through 0.22 μm membranes (Millipore, Bedfore, MA, USA).

Rotavirus infection of mice - The rotavirus inoculum consisting of cesium chloride-purified ECwt triple-layered particles (TLPs) was adjusted to produce the first diarrhoeal event at 24 h post-inoculation (h.p.i.). Different volumes (0.1, 1, 2 and 5 µL) from a stock preparation [7.8 x 10^7 focus forming units (FFU)/mL)] were diluted with PBS to reach 100 µL and then directly applied into the 10-12 day old mouse pharynx. The lowest stock volume (0.1 µL) was routinely used to produce diarrhoea at 24 h.p.i. To determine the proportion of intestinal epithelial cells (IEC) infected in each mouse following three days post-inoculation (d.p.i.), six mice were inoculated with ECwt TLPs whereas one mouse was left uninoculated as a control. The percentage of infected IEC was determined from the small intestinal villi isolated from virus-infected mice and uninfected control using the immunochemistry assay described below. The time-course of the percentage of infected IEC was determined by inoculating 10 mice with ECwt TLPs and slaughtering them at one-five d.p.i. The percentage of infected IEC was visualised on isolated intestinal villi by immunochemistry. Comparisons were made against cells from uninoculated control mice.

Immunochemistry assay - Small intestinal villi from infected mice were prepared for immunochemistry analysis as previously described (Guerrero et al. 2010). Cells were fixed with ice-cold methanol acetic acid (3:1) for 20 min and then place onto coverslips. After drying, endogenous peroxidase was inactivated by treatment with 3% H_2O_2 in 50% methanol. Fixed cells were reacted with rabbit polyclonal Abs (produced in our animal facilities) against rotavirus SPs or non-structural proteins NSP4 or NSP5. A horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ab (Santa Cruz Biotechnology Inc) was used as secondary Ab. Peroxidase activity was revealed with aminoethylcarbazole (AEC) substrate (Sigma, St. Louis, MO, USA). Small intestinal villi from infected and drug-treated mice were submitted to the same procedure as well as those from non-infected control mice. Ten representative fields were photographed at 40X magnification and the proportion of positive cells to infection was expressed as mean percentage relative to the total recorded cells.

ELISA - Capture ELISA was used for determining the accumulation of viral antigen in intestinal villi from infected mice which were either treated with drugs or left untreated. Procedures were essentially as those previously described (Guerrero et al. 2010). Briefly, villi preparations isolated from the entire small intestine were gently agitated immediately before taking samples for assay to ensure homogeneity in villi composition. Isolated villi samples containing about 10 mg/mL were lysed in modified radio immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tritonin X-100, 1% NP-40, 0.5% deoxycholate) and subjected to centrifugation. The 2,500 *g* supernatant was applied to ELISA plate wells previously coated with polyclonal guinea pig Abs (1:1000) against rotavirus SP. After PBS washing, polyclonal rabbit anti-rotavirus SP Abs (1:3000) were added and after washing away unbounded Abs with PBS, HRP-conjugated donkey anti-rabbit IgG secondary Abs (0.08 µg/mL Santa Cruz Biotechnology Inc) (SC-2313) were added. The reaction was revealed using o-phenylenediamine dihydrochloride in stable peroxidase buffer (Pierce, Rockford, IL, USA). Optical density $(OD)_{492\,nm}$ was determined using an ELISA plate reader. Background OD values from uninfected villus lysates were subtracted from all infected samples. The cut-off value was calculated as the mean plus two standard deviations of the negative control wells. ECwt TLPs were used as an internal positive control. Expression of Hcs70 and PDI in intestinal villi isolated from ECwt infected mice which had or had not been treated with NAC (18 mg/kg/day) were also analysed by ELISA.

Intestinal villus isolation from infected mice - A previously described procedure for the small intestinal villus isolation was used (Guerrero et al. 2010). Briefly, entire small intestines were flushed with ice-cold minimum essential medium (MEM) containing 1.5 mM ethylenediamine tetraacetic acid (EDTA) and antibiotic/antimycotic solution before being opened longitudinally and cut into 0.5-cm-long segments. Following incubation and agitation in the same medium at 37ºC for 15 min, the cell/tissue preparation was further dispersed by gentle pipetting and immediately passed through a sterile metal net (1 mm^2). After the dispersed preparation was filtered twice, the filtered product was collected by low centrifugation to be subsequently resuspended and washed in MEM containing antibiotic/antimycotic solution. The intestinal villus-enriched preparation was used for recording the percentage of ECwt infected cells and the in vitro titration of virus infectivity.

Virus purification - Small intestines isolated from ECwt infected mice were homogenised with MEM containing antibiotic/antimycotic solution and submitted to a freeze-thaw cycle before 1,1,2-trichlorotrifluoroethane (Sigma, St. Louis, MO, USA) extraction and sucrose/cesium chloride gradient centrifugation as previously described (Gualtero et al. 2007). Visible virus bands were collected by aspiration with a syringe, diluted with tris buffered saline (TBS) (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM MgCl2, 5 mM CaCl2) and collected by high speed centrifugation (150,000 *g* for 1.5 h at ºC) prior to resuspension in TBS. Virus infectivity was titrated in vitro on isolated intestinal villus (Guerrero et al. 2010) using an immunochemistry assay as previously described.

Drug treatment of infected mice - Once the ECwt infected mice showed the first diarrhoeal episode, they were orally administered three daily doses (100 µL) for three days of one of the following drugs: NAC (1200, 600, 300, 150, 75, 37, 18, 9, 4 and 2 mg/kg/day), DCF (1 mg/kg/day), IBF (20 mg/kg/day), AA (20 mg/kg/day), PGZ (30 mg/kg/day) or RGZ (4 mg/kg/day). Three mice were used for each dose and each experiment was performed twice. After the three-day drug treatment, the mice were killed and their small intestine processed for immunocytochemistry analysis of the mean percentage of virus-infected villus cells. Intestinal villi from

uninfected mice administered with PBS and ECwt infected mice treated with PBS were used as controls. The specificity of NAC inhibitory effect on ECwt infection was determined by infecting comparable ICR mice with purified human reovirus type 1 (Sharpe et al. 1978) using two oral inoculations (100 µL; 8 x 10^4 FFU/mL) separated by 12 h. Following the first diarrhoeal episode (approximately 48 h.p.i), reovirus-infected mice were orally administered NAC (18 mg/kg/day) three times a day for three days. Six hours after the last dose, the mice were killed and their small intestinal villi isolated for immunochemistry analysis of the percentage of virus infected cells. Anti-rotaviral effect of NAC and NTZ were compared by treating ECwt infected mice with NAC (18 mg/kg/day) or NTZ (7.5 mg/kg/day) as described above. Antiviral effect was determined by immunocytochemistry analysis in terms of the mean percentage of virus infected villus cells relative to villus cells from uninfected and drug-untreated control mice.

Expression of COX, ERp57, Hsc70, NF-κB, Hsp70, PDI and PPARγ in villus cells from infected mice - Following three d.p.i., expression of COX, ERp57, Hsc70, NF-κB, Hsp70, PDI and PPARγ was assessed by indirect immunofluorescence in cross-sections of mouse small intestines or flow cytometry analysis of IEC. Small intestine cross-sections (1 cm) were fixed in 10% paraformaldehyde in PBS and embedded into paraffin wax. Paraffin-embedded sections (3-5 µm) were placed onto coverslips, deparaffinised in xylene and rehydrated in a graded ethanol series. Endogenous peroxidase activity was quenched by treating sections with 3% H_2O_2 in 50% methanol for 15 min and then cells were permeabilised with 1% sodium dodecyl sulfate (SDS) for 5 min. After washing with PBS, non-specific binding was blocked with 1% bovine serum albumin (BSA) for 15 min. ECwt infection was assessed by applying rabbit polyclonal anti-rotavirus SP Ab to the sections followed by PBS washing and addition of HRP-conjugated goat anti-rabbit secondary Ab. Immunostaining was visualised using AEC chromogen. The same coverslips were washed three times with PBS and then incubated with 50 mM NH_4Cl for 30 min. Sections were incubated with the first goat Ab against COX-2, ERp57, Hsc70, Hsp70, PDI, PPARγ (0.2 µg/mL; SC-1747, SC18619, SC1059, SC1060, SC17222, SC6285, Santa Cruz Biotechnology Inc) or rabbit Ab against NF-κB (0. 25 µg/mL; 51-3500, Zymed) in PBS containing 50 mM NH4Cl for 1 h at 37ºC. After three washes with PBS, fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat Ab (0.08 mg/mL; SC-2024, Santa Cruz Biotechnology Inc) or FITC-conjugated donkey anti-rabbit Ab (0.08 mg/mL; SC-2090, Santa Cruz Biotechnology Inc) diluted in PBS containing 1% BSA were added. Following PBS washing, sections were mounted in 70% glycerol in PBS and examined with a fluorescence microscope (VanGuard).

Flow cytometry analysis of COX-2, Hsc70, NF-κB, Hsp70, PDI or PPARγ expression was performed by fixing PBS/EDTA (5 mM)-isolated IEC from ECwt infected mice (3 d.p.i.) in methanol/acetic acid (3:1 v/v) for 1-4ºC before incubation with 50 mM NH_4Cl. Goat primary Abs against the above mentioned proteins and rabbit primary Abs against rotavirus SP were mixed together diluted in PBS containing 50 mM NH_4Cl and then added to IEC. Following three washes with PBS, FITC-conjugated mouse anti-goat IgG secondary Abs (0.88 µg/mL; SC-2356, Santa Cruz Biotechnology Inc) and phycoerythrin-conjugated anti-rabbit IgG Abs (0.88 µg/mL; SC-3753, Santa Cruz Biotechnology Inc) diluted in PBS supplemented with 50 mM NH_4Cl and 1% BSA were added. Immunofluorescence analysis was performed on a Cyan (Dako) flow cytometer using FlowJo software (Tree Star, Ashland, OR, USA). Flow cytometry analysis was also used for assessing Hsc70 and PDI expression in IEC isolated from ECwt infected mice (3 d.p.i.) which had or had not been treated with NAC (18 mg/kg/day).

For confocal microscopy analysis of Hsc70 and PDI expression, deparaffinised and rehydrated sections from intestines isolated from ECwt-infected mice that had or had not been treated with NAC (18 mg/kg/day), IBF (20 mg/kg/day), DCF (1 mg/kg/day) or PGZ (30 mg/kg/day) were incubated with primary goat anti-Hsc70 or anti-PDI Abs mixed together with rabbit anti-rotavirus SP Abs. After three washes with PBS, FITC-conjugated donkey anti-rabbit IgG Abs (0.88 µg/mL; SC-2090, Santa Cruz Biotechnology Inc) and Alexa Flour 568-conjugated donkey anti-goat IgG Abs (4.4 µg/mL; A-11057) (Invitrogen) diluted in PBS containing 1% BSA were added together to the sections. Subsequently, they were washed with PBS and the nuclei were counterstained with 4'-6-diamidino-2-phenylindole (0.5 µg/mL). Coverslips were mounted in 70% glycerol and images examined using a confocal microscope (Nikon C-1). Pearson's co-localisation analysis and image processing software Image J were used to assess the co-localisation of Hsc70, PDI and rotavirus proteins.

Western blotting (WB) - Analysis of the accumulation of both rotavirus structural and non-SPs in villi isolated from drug-treated ECwt-infected mice was performed by preparing villus lysates using sonication at 30% amplitude for 3 min with 30 sec sonication intervals in the presence of phenylmethanesulfonyl fluoride, followed by adjusting of protein concentration using a NanoDrop microscale spectrophotometer (NanoDrop® ND-1000). After WB, polyvinylidene fluoride (PVDF) membranes were probed with rabbit Abs against rotavirus SP mixed with rabbit Abs against NSP4 and NSP5, followed by treatment with HRP-conjugated goat anti-rabbit Abs and AEC substrate. The behaviour of Hsc70 and PDI expression in intestinal villus cells from ECwt-infected mice (3 d.p.i.) following drug treatment was further analysed using WB. Following the first diarrhoeal episode (about 24 h.p.i.), mice were treated with three daily doses of one of the following drugs: IBF (20 mg/kg/day), DCF (1 mg/kg/day), PGZ (30 mg/kg/day), RGZ (4 mg/kg/day), NAC (18 mg/kg/day), AA (20 mg/kg/day) or sodium diphenoxylate (7.5 mg/kg/day). Isolated intestinal villi were lysed in RIPA buffer and then sonicated as indicated above. Lysates were kept at -70ºC until used. Protein per gel lane was adjusted before separation in sodium dodecyl

sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10%) and transfer to PVDF membranes. The target proteins were probed with primary goat Abs against Hsc70 (0.2 µg/mL; SC-1059, Santa Cruz Biotechnology Inc) or PDI (0.2 µg/mL; 20132, Santa Cruz Biotechnology Inc). HRP-conjugated donkey anti-goat IgG Ab (0.4 µg/mL; SC-2020, Santa Cruz Biotechnology Inc) was used as a secondary Ab and the reaction was developed with intensified luminescence (Pierce) or AEC. Commercial Abs against COX-2, ERp57, Hsc70, Hsp70, PDI, PPARγ or NF-κB were monitored by WB for the absence of Abs against rotavirus proteins. Purified rhesus rotavirus (RRV) triple layered particles (TLPs) were subjected to SDS-PAGE/WB analysis using commercial preparations as primary Abs and the respective HRP-conjugates as secondary Abs and AEC substrate.

Statistical analysis - The difference in mean and homogeneity of variance of infectivity or antigen accumulation in cells was tested by one-way analysis of variance and the Student's *t* test. Results were considered statistically significant if the p value is under 0.01.

RESULTS

NSAIDs, PPARγ agonists and NAC treatment reduce ECwt infectivity in ICR mice - The cell viability of IEC of small intestinal villi isolated from uninfected mice was estimated to be about 80% by the Trypan blue exclusion test. The inoculation of six mice with ECwt resulted in the infection of IEC as determined by immunochemistry analysis of small intestinal villi isolated from the entire small intestine. After three d.p.i., the proportion of IEC resulting positive for virus infection ranged from 57-95%, whereas the mean infectivity was about 75% (Fig. 1A). In all cases, this mean percentage was taken as 100% infectivity for control villi isolated from drug-untreated mice. The absence of differential ECwt infection for small intestine sections has been previously reported for isolated villi (Guerrero et al. 2010). To analyse the time-course of ECwt infectivity, two mice were killed every 24 h following virus inoculation. The results indicated that no significant differences were found in infectivity through four d.p.i. when using an ECwt inoculum consisting of 7.8 x 10^4 FFU/mL per mouse (Fig. 1B). The relatively low dispersion values observed for virus infectivity through the period examined suggested that three d.p.i. could be a suitable post-inoculation time for testing drug effects on infectivity. To test for NAC effect on ECwt infectivity, mice at 24 h.p.i. were administered 2 mg NAC/kg/day. After the three-day treatment, infectivity remained similar to that of untreated infected mice (Fig. 2A). Administration of NAC 4 mg/kg/day reduced infectivity to 55%, whilst NAC doses ranging from 9-1,200 mg/kg/day were able to reduce infectivity to 10-4% (Fig. 2A). This NAC dose range thus produced an infectivity reduction that fluctuated between 78.7-94.7%. In searching for a correlation between the percentage of infected cells and the total rotavirus structural antigen accumulated in IEC from isolated villi, an ELISA analysis was conducted. $OD_{492\ nm}$ from ELISA plate wells suggested a dose-dependent inhibitory effect at low NAC concentrations similar to that observed from immunochemistry assay. However, this inhibitory profile from ELISA values was not entirely maintained at higher NAC concentrations (Fig. 2A). The effect of other drugs such as NSAIDs (DCF, IBF), PPARγ agonists (PGZ, RGZ), AA and diphenoxylate-HCl on ECwt infectivity was determined by detecting the percentage of IEC resulting positive for rotavirus antigen. When using immunocytochemistry detection of SP, NSP4 or NSP5 as rotavirus antigen in isolated villi, the respective inhibitory effect was as follows: DCF, 35%, 27% and 23%, IBF, 90%, 90% and 93%, PGZ, 32%, 5% and 5%, RGZ, 25%, 20% and 20%, AA, 55%, 50% and 20% and NAC, 90%, 80% and 80% (Fig. 2B-D). The differential rotavirus antigen detection led to conclude that regardless of the virus antigen used for determining the percentage of infected cells, the inhibitory trend was similar for each drug tested. However, the highest inhibitory effects were obtained with administration of IBF and NAC.

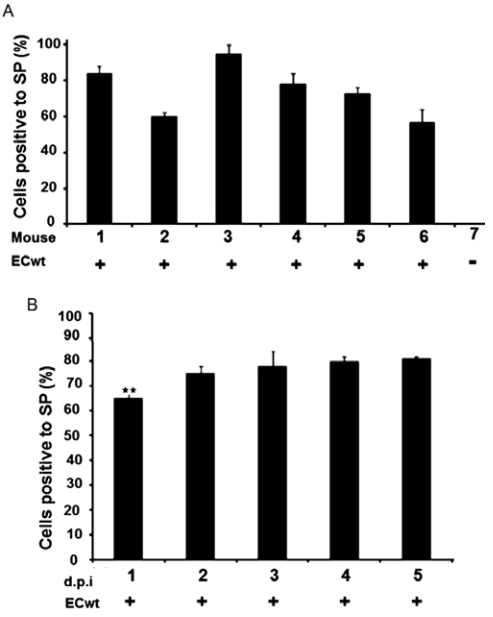

Fig. 1: proportion of ECwt-infected cells in small intestinal villi. A: ICR mice were inoculated with ECwt (8 x 10^4 focus forming units/mL) and their intestinal villi isolated from the entire small intestine after three days post-inoculation (d.p.i.) For immunochemistry analysis, villi were fixed with ice-cold methanol-acetic acid and reacted with rabbit polyclonal antibodies (Abs) against rotavirus structural proteins (SP). The reaction of villus-associated cells from different mice (1-6) was detected using horseradish peroxidase-conjugate goat anti-rabbit Ab and aminoethylcarbazole substrate. Graph shows significant ECwt infection as compared with uninfected control mice ($p < 0.0001$); B: small intestinal villi isolated at one, two, three, four and five d.p.i. from ECwt-infected mice (n = 10 mice distributed in 5 days) were fixed with methanol-acetic acid and treated with polyclonal Abs against rotavirus SP before detecting the reaction as in indicated in A. Non-infected mice were used as a control. Photographs from 10 representative fields were analysed for each experimental villus intestinal epithelial cells sample. Data are expressed as percentage ± standard deviation ($p < 0.002$) of villus-associated cells being positive to ECwt SP relative to the total cells analysed.

The specificity of NAC inhibition on rotavirus infection was tested by inoculating mice with human reovirus type 1 and then treating them with NAC. This drug treatment did not have any detectable inhibitory effect on reovirus infection as the percentage of infected villus cells remained about 66% which was quite similar to that (67%) found in reovirus infected mice without NAC treatment (Fig. 2E). In contrast, villus cells from ECwt infected mice without NAC treatment showed an infection of 95% which was reduced to 3% after NAC treatment. To compare the NAC inhibitory effect with that of NTZ (a drug used for treating rotaviral diarrhoea in children over 1 year of age), ECwt infected mice were treated with either drug following the first diarrhoeal episode. Villus cells from NAC-treated mice (18 mg/kg/day) showed a mean percentage infection of 3% after the three-day treatment as compared with villus cells from NTZ-treated mice (7.5 mg/kg/day) which showed a

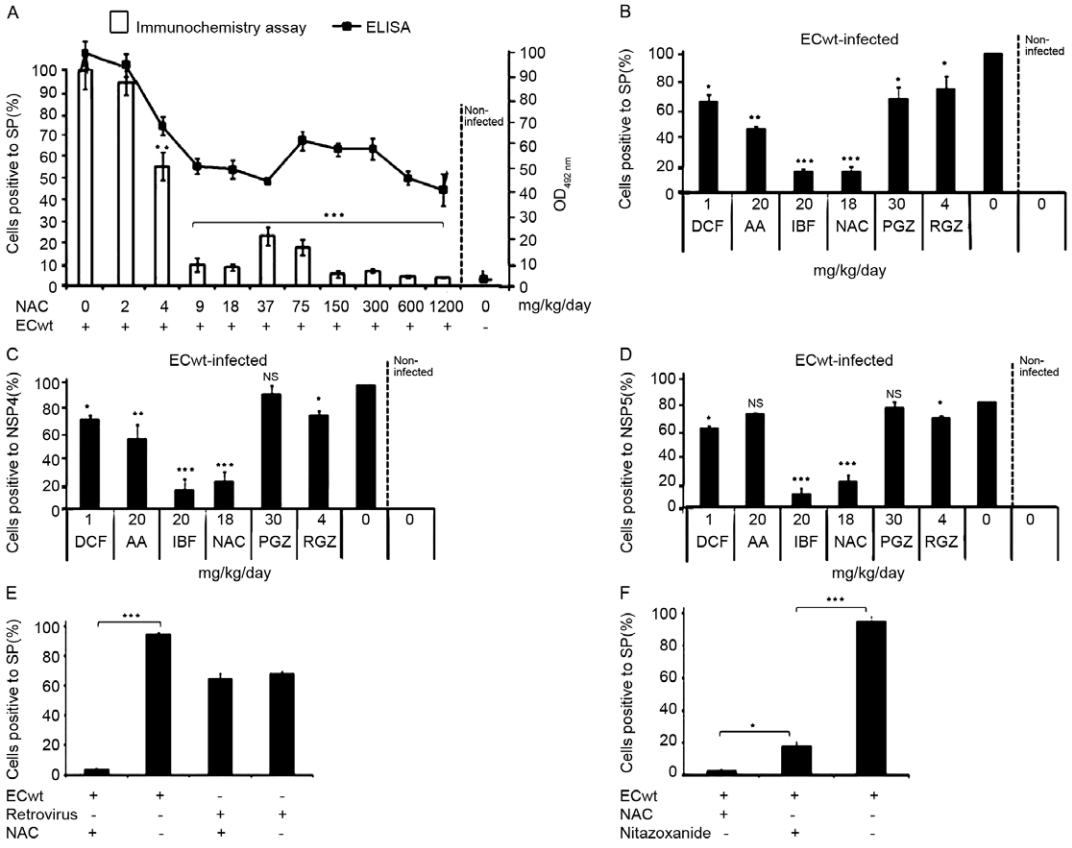

Fig. 2: effect of drug treatment on ECwt infection of villus intestinal epithelial cells (IEC). A: ICR mice were infected with ECwt and after 24 h post-inoculation (h.p.i.) mice were differentially subjected to treatment with increasing N-acetylcysteine (NAC) doses (n = 10 mice for each dose) during three days. Mouse small intestinal villi were isolated after three-day treatment and submitted to immunochemistry and ELISA analysis. Rabbit polyclonal antibodies (Abs) against rotavirus rotavirus structural proteins (SP), horseadish peroxidase (HRP)-conjugate goat anti-rabbit Ab and aminoethylcarbazole (AEC) substrate were used for immunochemistry analysis of methanol-acetic acid fixed villi. Guinea pig Abs against rotavirus SP for capturing rotavirus antigen and rabbit Abs against rotavirus SP for its detection were used for ELISA analysis of radio immunoprecipitation assay (RIPA) lysates from villus IEC. Reaction was revealed with HRP-conjugated goat anti-rabbit Ab and o-phenylenediamine dihydrochloride substrate and then read at optical density $(OD)_{492\ nm}$. Villi or RIPA lysates of villi from both ECwt-infected and uninfected mice were used as control. Graph shows significant NAC inhibitory effect ($p < 0.001$); B: mice were infected with ECwt and treated from 24 h.p.i. with different drugs (n = 6 mice for each drug) at the indicated doses during three days. Immunochemistry analysis of villi was conducted as indicated in A for detecting rotavirus SP. Villi from ECwt-infected mice that had not been treated with drugs were used as a control; C: procedures were as indicated in B, except that rotavirus non-structural proteins NSP4 was detected in the immunochemistry analysis; D: procedures were as indicated in B, except that rotavirus NSP5 was detected in the immunochemistry analysis. For results shown in A-D, villi isolated from either ECwt-infected or uninfected mice without treatment were used as a control. Results of drug treatment were found to be significant for B-D ($p < 0.01$); E: mice were infected with either ECwt or reovirus type 1 and after 48 h.p.i. mice (n = 4 mice for each experimental group) were treated with NAC (18 mg/kg/day) during three days. Rabbit Abs against reovirus SP, HRP-conjugated goat anti-rabbit Ab and AEC substrates were used for immunochemistry analysis of villi from reovirus infected mice. Graph shows significant NAC inhibitory effect ($p = 0.0003$); F: mice were infected with ECwt and after 24 h.p.i. mice (n = 4 for each experimental group) were treated with either NAC (18 mg/kg/day) or nitazoxanide (7.5 mg/kg/day) for three days. Graph shows significant NAC and nitazoxanide (NTZ) inhibitory effect in comparison to untreated infected control ($p = 0.0003$) and significant NAC inhibitory effect relative to NTZ treatment ($p = 0.007$). The immunochemistry analysis of villi was conducted as indicated in A for rotavirus SP. Data correspond to two independent experiments performed in duplicated and are expressed as percentage ± standard deviation (SD) of infected cells relative to total cells analysed in the case of the immunochemistry analysis or mean percentage ± SD of the rotavirus antigen present in RIPA lysates of villi from infected and NAC-treated relative to that from infected and untreated mice. AA: ascorbic acid; DCF: diclofenac; IBF: ibuprofen; PGZ: pioglitazone; RGZ: rosiglitazone.

mean percentage infection of 17%. Infection percentage for villus cells from control virus-infected mice without drug treatment was 95% (Fig. 2F).

The inhibitory effect of IBF, PGZ or NAC on ECwt infection was further investigated by assessing the viral antigen accumulation in villus cells from ECwt-infected mice after a three-day treatment following the first diarrhoeal event. ELISA results showed that the total ECwt antigen accumulated was reduced by about 82%, 76.4% and 100% when mice were treated with IBF, PGZ and NAC, respectively. ELISA results were expressed as mean $OD_{492\,nm}$ values after subtracting mean background values of villus cell lysates from non-infected mice (Fig. 3A). Similar results were obtained after analysing proteins of villus cell lysates from virus-infected mice that had been treated with IBF or PGZ. Western analysis of lysate proteins led to conclude that both drugs drastically reduced the accumulation of both SP and NSP in cells from drug-treated mice as compared with samples from untreated control mice (Fig. 3B). The inhibitory effect of the above drugs, together with that from AA, DCF, RGZ and diphenoxylate was further confirmed by immunocytochemistry analysis. As shown in the representative images in Fig. 3C, drug treatment resulted in considerable reduction of cells positive for Abs against rotavirus SP when mice were treated with AA, IBF or NAC. An inhibitory effect, although lesser in degree, was observed in cells from virus-infected mice that were treated with RGZ, PGZ or DCF (Fig. 3C).

The effectiveness of the NAC inhibition of ECwt infectivity depends of the frequency of NAC application and the post-infection time of treatment - The NAC-me-

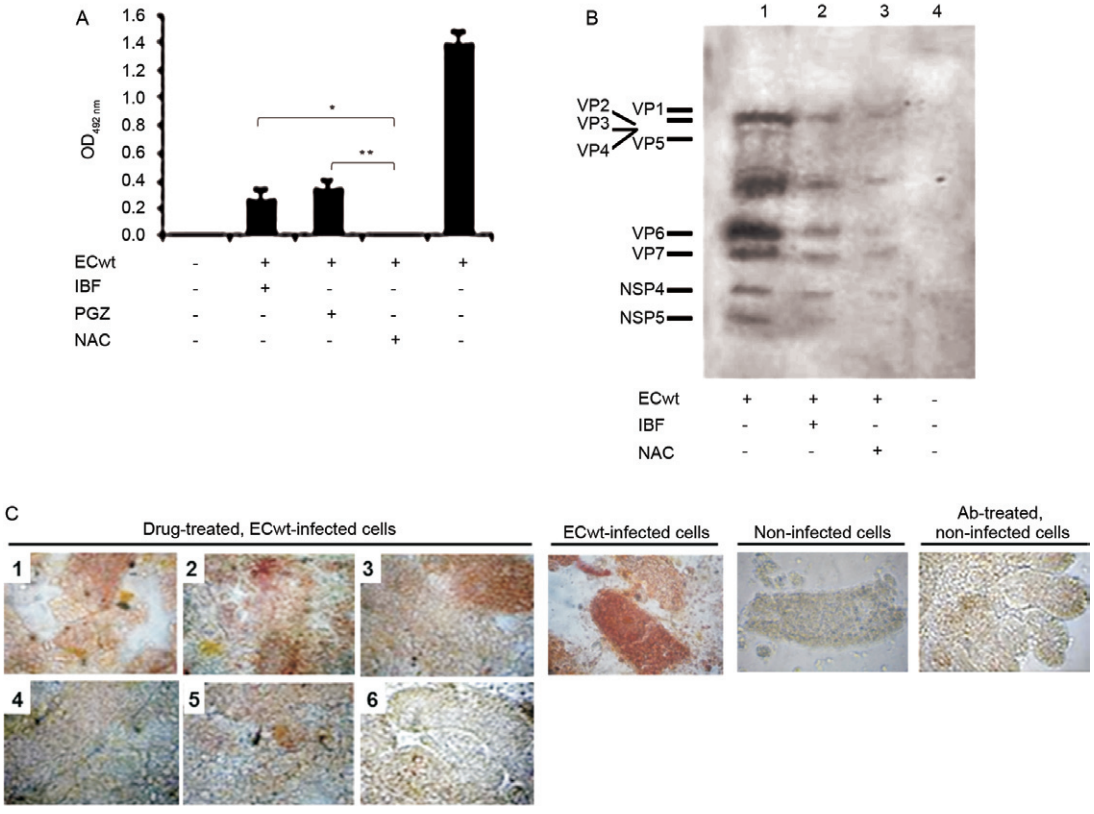

Fig. 3: effect of drug treatment on the accumulation of rotavirus structural proteins (SP) in villus cells. A: ECwt-infected mice (n = 4 mice for each experimental group) were treated after 24 h post-inoculation with ibuprofen (IBF) (20 mg/kg/day), pioglitazone (PGZ) (30 mg/kg/day) or N-acetylcysteine (NAC) (18 mg/kg/day) during three days and then their small intestinal villi were isolated and lysed with radio immunoprecipitation assay (RIPA). Guinea pig antibodies (Abs) against rotavirus SP for capturing rotavirus antigen and rabbit Abs against rotavirus SP for its detection were used for ELISA analysis of RIPA lysates from villi. Reaction was revealed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ab and o-phenylenediamine dihydrochloride substrate and then read at optical density $(OD)_{492\,nm}$. Rotavirus antigen was expressed as mean $OD_{492\,nm}$ ± standard deviation. Graph shows significant NAC inhibitory effect relative to IBF or PGZ treatments ($p < 0.002$); B: mice were infected with ECwt and treated with IBF (20 mg/kg/day) or NAC (18 mg/kg/day) during three days. Small intestinal villi were isolated and lysed using sonication. Cell lysates (75 µg protein/well) were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis/Western blotting using rabbit Abs against rotavirus SP, non-structural proteins NSP4 and NSP5, HRP-conjugated goat anti-rabbit Abs and aminoethylcarbazole (AEC) substrate. Cell lysates from ECwt-infected mice without drug treatment (Lane 1), cell lysates from ECwt-infected mice treated with IBF (Lane 2), cell lysates from ECwt-infected mice treated with NAC (Lane 3), cell lysates from uninfected mice (Lane 4); C: small intestinal villi were isolated from ECwt-infected mice that have been treated with rosiglitazone mg/kg/day) (1), PGZ (30 mg/kg/day) (2), diclofenac (1 mg/kg/day) (3), ascorbic acid (20 mg/kg/day) (4), IBF (20 mg/kg/day) (5) or NAC (18 mg/kg/day) (6) during three days. Villi were submitted to immunochemistry analysis using rabbit polyclonal Abs against rotavirus SP, HRP-conjugate goat anti-rabbit Ab and AEC substrate. Representative images are shown. Data are from three independent experiments using three mice per experiment. VP: rotavirus structural proteins.

diated inhibition of ECwt infectivity shown above was further investigated in terms of its dependence on the frequency of dose application. When NAC was administered at only one dose of 6 mg/kg/day following the first diarrhoeal episode at about 24 h, the percentage of ECwt infected villus cells was 68% after 4 h from the application, whereas the percentage for virus infected and NAC-untreated control cells was 69%. Administration of 6 mg NAC/kg every 8 h for one day (18 mg/kg/day) or for two days, which corresponded to three-six total applications, respectively, led to a reduced proportion of infected cells (34%) in comparison to the percentage observed in NAC-untreated control cells (Fig. 4A). Maintaining the same dose but during three (9 applications) or four (12 applications) days resulted in a considerable decrease of the percentage of virus infected villus cells (4%) when comparison was made with that of cells from NAC-untreated control mice (79%). Monitoring the dose frequency effect on NAC inhibition using ELISA for the accumulation of viral antigen in villus cells led to an inhibition profile that was similar to that found using the immunochemistry assay (Fig. 4A). These results suggested that NAC ability to inhibit ECwt infectivity in villus cells increased with increasing the frequency of dose applications.

To determine the post-inoculation time at which NAC was able to exert its inhibitory effect, mice were treated with NAC (18 mg/kg/day distributed in three daily applications) after 24, 48, 72 and 96 h.p.i. This means that each mouse received 12, 9, 6 and three applications, respectively. Six hours after the last application, the proportion of villus cells from mice treated with NAC after 24 and 48 h.p.i. resulting positive to ECwt infection was 15% and 23%, respectively, whereas the percentage for cells from NAC-untreated control mice was 74% (Fig. 4B). Starting NAC treatment after 72 h.p.i. resulted in 60% of villus cells resulting positive to viral infection, whilst NAC application after 96 h.p.i. led an infection percentage that was statistically similar to that of cells from untreated control mice (Fig. 4B). The inhibition profiles of NAC application at different post-inoculation times were essentially similar irrespective of whether immunochemistry assay or ELISA were used for measuring virus infection (Fig. 4B).

ECwt infection of ICR mice increased expression of COX-2, ERp57, Hsc70, NF-κB, Hsp70, PDI and PPARγ in intestinal villus cells - Some of the molecular mechanisms involved in the action of the drugs used above have previously been studied (Furuya et al. 2008, Rao & Knaus 2008, Gupta et al. 2010). In addition, there is some previous knowledge about the implication of certain cellular molecules in the rotavirus infection process (Lopez & Arias 2006, Calderon et al. 2012). To get insight into the mechanisms supporting the antiviral action of AA, IBF, DCF and NAC, their impact on the expression of COX-2, ERp57, Hsc70, NF-κB, Hsp70, PDI or PPARγ was evaluated using immunochemistry, indirect immunofluorescence, ELISA, WB and confocal microscopy. It has been reasonably assumed that sera raised in animals susceptible to rotavirus infection may contain Abs against rotavirus. WB analysis for determining Abs directed against rotavirus proteins in commercially prepared sera showed the lack of rotavirus reactivity in the commercial sera used for detecting the above mentioned proteins (data not shown). Immunochemistry and immunofluorescence analyses of intestinal cross-sections from ECwt infected mice indicated that cells showing the strongest positive signals for viral antigen coincided with those resulting positive for above mentioned proteins (Fig. 5A). To further characterise the correlation of virus infection and expression of the above proteins, a flow cytometry analysis was conducted. As shown in Fig. 5B, a simultaneous increase for viral antigen and

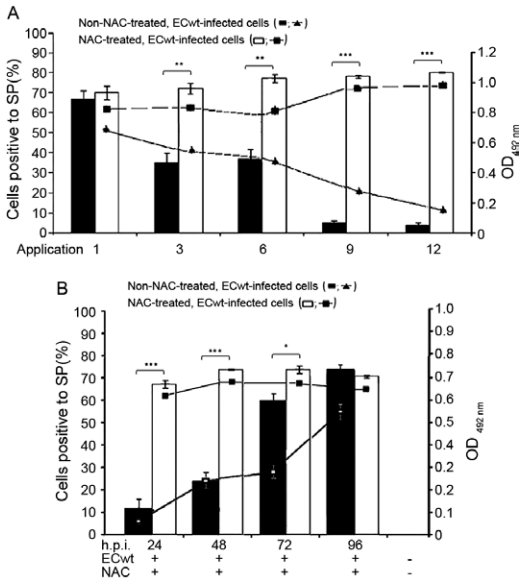

Fig. 4: effect of the frequency of dose application and post-inoculation time of application on the inhibition of ECwt infection by N-acetylcysteine (NAC). A: following the first diarrhoeal episode, ECwt-infected mice (n = 9 mice for each experimental group) were treated separately with one application of NAC (6 mg/kg/day) for one day or three applications (18 mg/kg/day) for one, two, three or four days, which corresponded to a total number of applications of one, three, six, nine or 12, respectively. Intestinal villi were isolated and analysed by immunochemistry using rabbit polyclonal antibodies (Abs) against rotavirus structural proteins (SP), horseradish peroxidase (HRP)-conjugate goat anti-rabbit Ab and aminoethylcarbazole substrate. Radio immunoprecipitation assay lysates from villi were analysed by ELISA using guinea pig Abs against rotavirus SP for capturing rotavirus antigen and rabbit Abs against rotavirus SP for its detection. Reaction was revealed with HRP conjugated goat anti-rabbit Ab and o-phenylenediamine dihydrochloride substrate before reading at optical density $(OD)_{492\ nm}$. Graph shows significant NAC inhibitory effect from three NAC applications ($p < 0.001$); B: ECwt-infected mice (n = 9 mice for each experimental group) were treated with NAC (18 mg/kg/day distributed in 3 daily applications) after 24, 48, 72 and 96 h post-inoculation (h.p.i.) which corresponded to a total number of 12, nine, six and three applications, respectively. Rotavirus SP accumulated in villus cells was analysed by immunochemistry and ELISA as indicated in A. Data are from three separate experiments using three mice per experiment. Data are expressed as mean percentage ± standard deviation (SD) of infected cells or mean $OD_{492\ nm}$ ± SD of rotavirus antigen. Graph shows significant inhibitory effect when NAC treatment started before 72 h following the first diarrhoeal episode ($p < 0.01$).

proteins COX-2, ERp57, Hsc70, NF-κB, Hsp70, PDI and PPARγ was observed in ECwt infected cells as compared with uninfected control cells.

To examine the effects of NAC treatment on the expression of Hsc70 and PDI in ECwt-infected villus cells, IEC isolated from mice that had been treated with NAC (18 mg/kg/day) during three d.p.i. were analysed by flow cytometry. The results showed that ECwt infection caused the induction of Hsc70 and PDI expression in comparison to control cells, whereas NAC treatment resulted in Hsc70 and PDI expression returning to levels similar to those observed in uninfected control cells (Fig. 6A). NAC effect on Hsc70 and PDI expression in ECwt infected mice was also analysed by ELISA. The analysis showed that HSc70 and PDI expression levels were increased in lysates of IEC from ECwt-infected mice, whereas NAC treatment returned these expression levels to values similar to those found in cell lysates from uninfected control cells (Fig. 6B). Induction of Hsc70 and PDI expression by ECwt infection was confirmed by SDS-PAGE/WB analysis of IEC lysates from infected and uninfected mice. This analysis showed that increased signals corresponding to Hsc70 and PDI protein bands were associated with ECwt infection as compared with IEC protein samples from uninfected control mice (Fig. 6C). Treatment of ECwt infected mice with NAC or IBF returned Hsc70 and PDI protein band intensity to a level similar to those observed in IEC protein samples of uninfected control mice (Fig. 6C). RGZ treatment seemed to induce a modest decrease of PDI from ECwt-infected IEC while apparently induced further increase of the Hsc70 expression level in IEC from infected mice. This latter effect was similarly observed in IEC samples from ECwt-infected mice that had been treated with PGZ (Fig. 6C). In addition, PGZ treatment of infected mice returned the PDI expression level to that observed for IEC samples from uninfected control mice (Fig. 6C). PDI and Hsc70 expression levels detected for IEC protein samples from ECwt-infected mice treated with DCF or AA were similar to those found in IEC protein samples from uninfected control mice (Fig. 6C). Except for the effect of PGZ and RGZ treatment on Hsc70 expression levels found in IEC samples from ECwt-infected mice, all drug treatments appeared to return PDI and Hsc70 expression levels to those observed for IEC protein samples from uninfected control mice.

Confocal microscopy analysis of cross-sections of small intestines from ECwt infected mice indicated that Hsc70 and PDI signals were increased in intestinal cross-sections from virus infected mice that had not been treat-

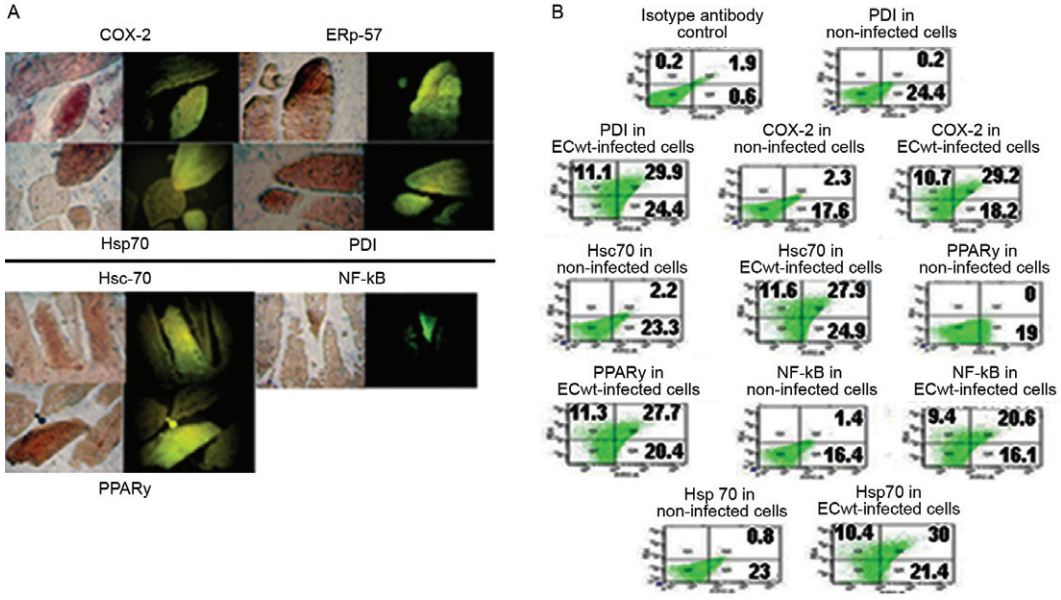

Fig. 5: increased expression of cyclooxygenase (COX)-2, ERp57, Hsc70, nuclear factor kappa B (NF-κB), Hsp70, protein disulphide isomerase (PDI) and peroxisome proliferator-activated receptor gamma (PPARγ) in villus cells infected by ECwt. Mice were infected with ECwt and at thre days post-inoculation (d.p.i.) their small intestinal villi were isolated. Villi were analysed by immunocytochemistry (A) or flow cytometry (B) (n = 2 mice for each analysis). A: the immunocytochemistry analysis of villus cross-sections was performed by using rabbit polyclonal anti-rotavirus structural proteins (SP) antibody (Ab), horseradish peroxidase-conjugated goat anti-rabbit secondary Ab and aminoethylcarbazole chromogen. Cross-sections used in immunocytochemistry were treated with 50 mM NH4Cl and then incubated with goat Ab against COX, ERp57, Hsc70, Hsp70, PDI, PPARγ or rabbit Ab against NF-κB. Fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat Ab or FITC-conjugated donkey anti-rabbit Ab were added and sections were examined with a fluorescence microscope (VanGuard); B: flow cytometry analysis of COX, Hsc70, NF-κB, Hsp70, PDI o PPARγ expression was performed in intestinal epithelial cells (IEC) isolated from infected villi from ECwt-infected mice. Methanol/acetic acid-fixed IEC were incubated with goat primary Abs against the above mentioned proteins mixed with rabbit primary Abs against rotavirus SP. FITC-conjugated mouse anti-goat IgG Abs and phycoerythrin-conjugated anti-rabbit IgG Abs were used as secondary Abs. Immunofluorescence analysis was performed on a Cyan (Dako) flow cytometer using a FlowJo software. All assays were performed at least twice.

ed with drugs, whilst NAC treatment led to decreased signals of Hsc70 and PDI, which were comparable to those observed in samples from uninfected mice (Supplementary data). After quantification of co-localisation using the Pearson's co-localisation coefficient, it was also found that viral antigen in the villus cells did not co-localised with Hsc70 or PDI (Supplementary data). PGZ also decreased Hsc70 and PDI signals to levels similar to those observed with the NAC treatment (Supplementary data), while IBF treatment caused decreased signals for these proteins, but to a lesser extent than that found with NAC treatment (Supplementary data).

DISCUSSION

The two recently introduced live-attenuated rotavirus vaccines have been licensed in various countries for widespread use in massive vaccination programs (Nelson & Glass 2010, Santosham 2010). However, specific antiviral therapy for rotavirus disease is absent. Oral rehydration therapy has been useful for treatment of this disease and reduction of mortality, but without having a significant effect on the course of diarrhoea. Alternative, low cost and accessible developments for prophylactic and therapeutic treatment of the rotavirus-associated diarrhoeal disease are desirable and needed. In the present study, the inhibitory capacity of NAC, DCF, IBF, PGZ and RGZ on ECwt infection of ICR mice has been demonstrated. NAC treatment of infected mice immediately after the first diarrhoeal episode has occurred showed that rotavirus infection process can be interfered with once it has been established. On the other hand, NAC three-day treatment using a dose of 18 mg/kg/day was able to reduce virus infectivity by 80-97% as determined by immunocytochemistry analysis of isolated intestinal villi. However, when infection was determined in terms of rotavirus structural antigen accumulation in intestinal villi, ELISA analysis of lysates from villi showed that NAC treatment reduced viral antigen by 56-100%. The apparent discrepancy found between the immunochemistry and ELISA calculation of the proportion for the NAC inhibitory effect could be reflecting not only the different number of mice used in each experiment, but also the outbred condition of ICR mice. However, what should be highlighted from our study is that the NAC inhibitory effect was significantly produced on ECwt infection as compared with untreated virus-infected con-

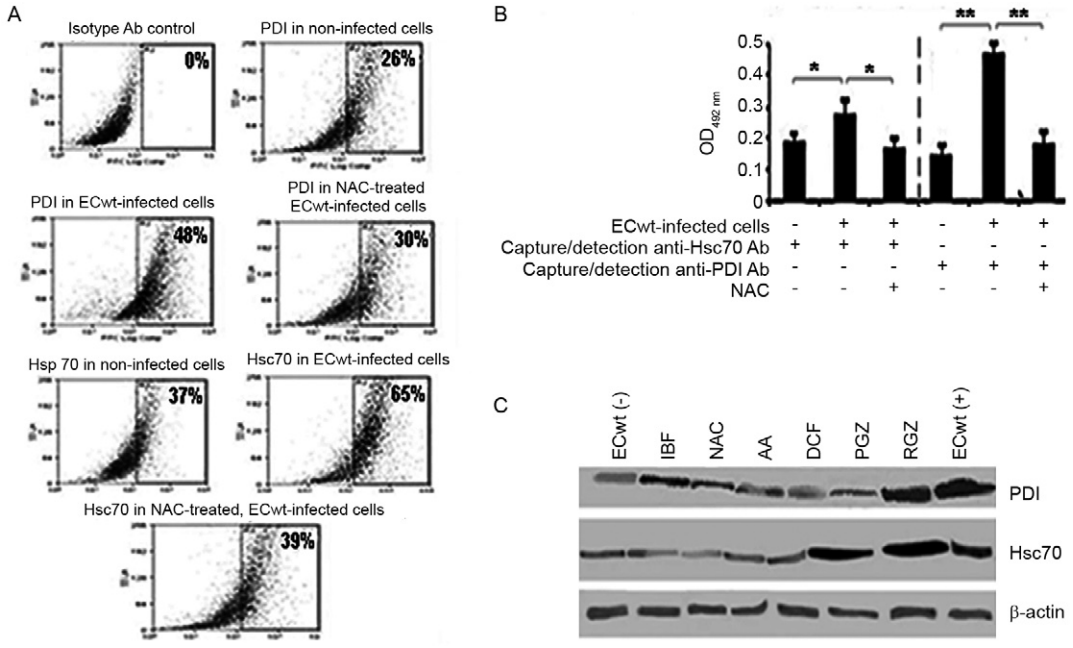

Fig. 6: effect of drug treatment on Hsc70 and protein disulphide isomerase (PDI) expression in small intestinal villi isolated from ECwt-infected mice. A: flow cytometric analysis was performed on intestinal epithelial cells (IEC) isolated from villi from ECwt-infected mice (n = 2 mice for each experimental group) that had been treated with ibuprofen (IBF) (20 mg/kg/day), diclofenac (DCF) (1 mg/kg/day), pioglitazone (PGZ) (30 mg/kg/day), rosiglitazone (RGZ) (4 mg/kg/day), N-acetylcysteine (NAC) (18 mg/kg/day), ascorbic acid (AA) (20 mg/kg/day) or difenoxilate sodium (7.5 mg/kg/day) during three days post-inoculation (d.p.i.). Goat primary antibodies (Abs) against Hsc70 or PDI and fluorescein isothiocyanate (FITC)-conjugated mouse anti-goat IgG secondary Abs were used. Immunofluorescence analysis was performed on a Cyan (Dako) flow cytometer using a FlowJo software; B: radio immunoprecipitation assay lysates from villi isolated from ECwt-infected mice (n = 3 mice for each experimental group) that had been treated with NAC (18 mg/kg/day) during three days after 24 h post-inoculation. were added to ELISA plates coated with rabbit polyclonal Abs against Hsc70 or PDI. Goat Abs against Hsc70 or PDI were used as detection Abs and the reaction was revealed using horseradish peroxidase (HRP)-conjugated donkey anti-goat Abs and phenylenediamine dihydrochloride substrate before reading at optical density $(OD)_{492\ nm}$. Lysates from uninfected villi were used as a control. Data are expressed as mean $OD_{492\ nm}$ Hsc70 or PDI antigen. Graph shows significant increase of Hsc70 and PDI expression ($p < 0.01$) and their significant reduction by NAC treatment ($p < 0.01$); C: cell lysates (75 µg protein/well) from villi isolated from ECwt-infected mice that had been treated with drugs as indicated in A were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis/Western blotting. Membranes were probed with goat anti-Hsc70 or PDI Abs and then with HRP-conjugated donkey anti-goat IgG Ab. B-actin was used as protein loading control. The reaction was developed with intensified luminescence (Pierce). Cell lysates from ECwt-infected or uninfected villi were used as a control.

trol mice regardless of the method used for determining virus infection. It is interesting to note that in the absence of NAC treatment, ECwt infection was maintained in about 75% of villus cells at least through the six d.p.i. tested. ECwt appears to be unable to infect 100% of villus cells during this post-infection period. These may be because mature enterocytes from the villus apex seem to be more susceptible to rotavirus infection than those located in the villus base (Pearson et al. 1978, Pearson & McNulty 1979). Although an estimate of the number of diarrheal episodes would have been a very useful variable in our work, we were unable to obtain a reliable and quantitative recording of the number diarrheal episodes of suckling mice even upon gentle abdominal palpation. This fact prompted us to use the villi isolated from small intestine from ECwt-infected mice as an approach to quantify rotavirus infection.

In searching for an explanation for the inhibitory activity exhibited by NAC, it should be noted that NAC has been shown to be a potent inhibitor of NF-κB activation in vascular endothelial cells (Schubert et al. 2002). NAC and other antioxidants have been reported to inhibit hydrogen peroxide-induced NF-κB activation (Gupta et al. 2010). The central role played by NF-κB signal pathway in physiological and pathological conditions has made it a potential target for pharmacological intervention (Magné et al. 2006, Gupta et al. 2010). On the other hand, some rotavirus strains have been shown to activate NF-κB (Rollo et al. 1999, Halasz et al. 2008, Holloway et al. 2009, Bagchi et al. 2010). Inhibition of rotavirus infectivity in MA104 cells has recently been observed in the presence of NAC (Guerrero et al. 2012). Despite these previous findings, further experimental work remains to be done in order to assess the implication of NF-κB activation during rotavirus infection of intestinal villus cells and the NAC contribution in interfering with this particular signal pathway.

The administration of IBF showed to have a high inhibitory capacity on virus infection being comparable to that of NAC, whereas DCF administration resulted in a much lower inhibitory activity, but slightly higher than that observed for RGZ. It is worth noting the differential inhibitory effect exhibited by both NSAIDs tested as IBF caused virus infectivity reduction by about 90% compared with only 35% for DCF. This might suggest that these NSAIDs use different mechanisms for interfering with rotavirus infection process in villus cells. Indomethacin, a NSAID, has proven to be effective in inhibiting rotavirus infection in Caco-2 cells where PKA-mediated ERK1/2, p38 MAPK and NF-κB pathways have been found to be involved (Rossen et al. 2004). IBF and indomethacin have been found to have PPARγ agonistic properties whereas IBF is known for its inhibitory effects on NF-κB activation which are absent in indomethacin (Poligone & Baldwin 2001, Youssef & Mostafa 2004, Little et al. 2007). The two PPARγ agonists assayed, PGZ and RGZ, showed relatively low inhibitory effect which was about 32% and 25%, respectively. These results might lead to the assumption that PPARγ activation is less involved in the mechanisms used by the rotavirus infection process in villus cells. Moreover, the inhibitory activity caused by AA might be related with the implication of redox reactions during the ECwt infection process. Several studies have demonstrated the implication of redox imbalance in the establishing of viral infections and the progression of virus-induced diseases (Beck et al. 2000).

To unravel whether NAC inhibitory action has some specificity for rotavirus infection, mice were infected with human reovirus type 1, also a member of the family Reoviridae, but differing in several respects from rotavirus (Roy 2006). As a result, NAC treatment did not bring about any inhibitory effect on reovirus infection. This finding suggested that the rotavirus inhibiting effect of NAC has some specificity at least for the rotavirus strain used to infect ICR mice. In order to figure out whether NAC has inhibitor capacity similar to that of NTZ, ECwt-infected mice were treated with either inhibitor. As indicated in Results section, NAC treatment had higher inhibitory activity than that seen for NTZ treatment. Although the NTZ dose used was equivalent to that used in children for treating rotavirus infection (Bailey & Erramouspe 2004, Rossignol et al. 2006), this result should be taken with caution since a more systematic study should be performed in which several NTZ concentrations are used in order to confirm this result. Taking into account that the highest frequency of rotavirus infection is found in children between six-24 months of age and infants under six months of age show the next highest frequency (Brandt et al. 1979, Crawley et al. 1993, Carraro et al. 2008), NAC could have an additional advantage over NTZ as the former can be used even in pre-term new-born infants (Ahola et al. 1999), whilst the latter may only be administered to children older than one year of age (Bailey & Erramouspe 2004, Rossignol et al. 2006).

Although PGZ was about three times less effective than IBF and NAC in its inhibitory capacity when virus infectivity was measured as percentage of infected villus cells, the PGZ inhibitory effect was similar to that of IBF when infection was quantified as viral antigen accumulated in villus cells. Besides the higher sensitivity of ELISA in detecting viral antigen, the question arises whether the inhibitory capacity shown by these drugs involves both the number of infected cells and the amount of viral antigen accumulated per infected cell. It appears to be that when comparing PGZ and IBF inhibitory activities IBF treatment would lead to a lower number of infected cells, but containing more viral antigen per cell than that present in the higher number of infected cells remaining after PGZ treatment. None of these possibilities was systematically addressed in the present study and therefore it can only be hypothesised about the probable incidence of these differential effects of drug treatment on virus infected cell percentage and virus antigen accumulated per infected cell. It should be pointed out that ELISA provides a quantitative and objective method for measuring specific antigens whereas immunochemistry staining is only semi-quantitative at best and is also subjected to the evaluator's bias. Furthermore, the decreased viral antigen presence in villus cells from NAC or IBF treated mice was also confirmed by WB. These findings

suggest that viral antigen is on average reduced by drug treatment when the antigen is considered in relation to all villus cells, which, when taken together with the percentage of infected cells, leaves open the question about the possible differential inhibitory impact of drugs on either virus entry or virus-directed protein synthesis or both.

The NAC inhibitory effect was frequency-dependent as 18 mg/kg/day distributed in three applications during three-day treatment was more effective than less frequent treatment schedules. In addition, NAC administration as early as the occurrence of the first diarrhoeal episode may allow a more successful therapeutic result. However, experiments aimed at determining the specific stage of viral life cycle affected by NAC were not conducted. It remains to be determined whether NAC had any inhibitory effect by directly contacting the enterocyte luminal surface following oral drug delivery or whether its inhibitory effect was more related with the amount of drug absorbed into the enterocytes from the lumen. Determining the half maximal effective concentration for NAC requires knowledge about its return kinetics from the systemic circulation back to the small intestinal cells either as NAC itself or cysteine. Nevertheless, NAC effects on rotavirus infection might be discussed within the context of signalling pathways affected by ROS, NSAIDs and PPARγ agonists.

In some cases, viral infections can cause inhibition of host protein synthesis whilst in other cases a virus-induced differential expression of host proteins has been found. ECwt infection of villus cells induced the expression of host-encoded proteins COX-2, ERp57, Hsc70, NF-κB, Hsp70, PDI and PPARγ as detected in cross-sections from intestinal villi. It deserves to be mentioned that Hsc70 and PDI have been identified as rotavirus-interacting proteins during the infection of MA104 cells (Guerrero et al. 2002, Calderon et al. 2012), whereas COX-2 has been found to be required and induced by rotavirus infection of Caco-2 cells (Rossen et al. 2004). On the other hand, the influenza virus-induced ER stress has been associated with increased levels of ER chaperone ERp57 (Roberson et al. 2012), whilst recruitment of Hsp70 chaperones has been found to be an important component of viral survival strategies (Mayer 2005). Some viruses benefit from the anti-apoptotic properties of NF-κB whose activation allows enhancement of viral replication, host cell survival and evasion of host immune responses (Hiscott et al. 2001). However, in the case of NF-κB, it should be specified in our study that since its detection was performed using an Ab directed against one of the NF-κB subunits (p50), its level does not reflect the activity of the factor. More detailed analyses are needed to determine whether the protein changes induced by ECwt infection of villus associated cells are truly components of a specific cellular response to rotavirus infection or are reflecting a general stress response. It is worth noting that NAC treatment of ECwt-infected mice returned the Hsc70 and PDI expression levels to values similar to those found in uninfected villus-associated cells. However, evidence is not provided that the effective NAC-inhibitory effect is specifically mediated by the NAC-induced decrease of Hsc70 and PDI expression. Therefore, further study is needed to unravel the complete role of stress-inducible proteins in rotavirus infection.

Taking together the present results, it appears to be that rotavirus infection benefits from inducing both oxidative stress and activation of proinflammatory signalling pathways from host epithelial cells lining the villi since the treatment of rotavirus-infected mice with NAC, NSAIDs or PPARγ agonist led to significantly reduced infection of villus cells. The results described here suggest the possibility of designing preventive and therapeutic strategies aimed at interfering with rotavirus infection of children or young zootechnic species.

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