Enteroaggregative *Escherichia coli* strain in a novel weaned mouse model: exacerbation by malnutrition, biofilm as a virulence factor and treatment by nitazoxanide

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Enteroaggregative *Escherichia coli* (EAEC) is increasingly recognized as a common cause of diarrhoea in healthy, malnourished and immune-deficient adults and children. There is no reproducible non-neonatal animal model for longitudinal studies of disease mechanism or therapy. Using two strains of human-derived EAEC to challenge weaned C57BL/6 mice, we explored an *in vivo* model of EAEC infection in mice, in which disease was monitored quantitatively as the growth rate, stool shedding and tissue burden of organisms; nutritional status was varied, and a new class of therapeutics was assessed. A single oral challenge of EAEC strain 042 resulted in significant growth shortfalls (5–8 % of body weight in 12 days), persistent shedding of micro-organisms in stools [$>10^{3.2}$ c.f.u. (10 mg stool)⁻¹ for at least 14 days] and intestinal tissue burden [$\sim 10^3$ c.f.u. (10 mg tissue)⁻¹ detectable up to 14 days post-challenge]. Moderate malnourishment of mice using a 'regional basic diet' containing 7 % protein and reduced fat and micronutrients heightened all parameters of infection. Nitazoxanide in subMIC doses, administered for 3 days at the time of EAEC challenge, lessened growth shortfalls (by >10 % of body weight), stool shedding [by 2–3 logs (10 mg stool)⁻¹] and tissue burden of organisms (by >75 % in the jejunum and colon). Thus, weaned C57BL/6 mice challenged with EAEC is a convenient, readily inducible model of EAEC infection with three highly quantifiable outcomes in which disease severity is dependent on the nutritional status of the host, and which is modifiable in the presence of inhibitors of pyruvate ferredoxin oxidoreductase such as nitazoxanide.

Received 19 April 2012

Accepted 3 March 2013

INTRODUCTION

Diarrhoeal illnesses are a major cause of child mortality worldwide (Black *et al.*, 2010). Enteroaggregative *Escherichia coli* (EAEC) has been recognized as a common cause of diarrhoea in both children and adults (Huang *et al.*, 2006b; Nataro, 2005). This bacterium was first characterized as a putative enteroadherent-aggregative pathogen by Vial *et al.* (1988). Diarrhoea has been reported in humans infected with EAEC strains JM221 and 042 (Mathewson *et al.*, 1986; Nataro *et al.*, 1995). In patients, EAEC causes a mucoid,

persistent or watery diarrhoea, which may contain faecal leukocytes, or leukocyte markers, and which can be especially severe in very young children and infants (Jiang *et al.*, 2003; Kermani *et al.*, 2010; Steiner *et al.*, 1998), as well as in immunodeficient individuals (Huang *et al.*, 2006a). In two separate studies, EAEC was found to be the second most common cause of travellers' diarrhoea (Shah *et al.*, 2009; Taylor *et al.*, 2006), and has been noted to be the leading bacterial cause of diarrhoea in Baltimore and New Haven in the USA (Nataro *et al.*, 2006).

Malnutrition, which has long-term effects on child growth and development (Guerrant *et al.*, 2008), has been associated with recurrent diarrhoea and infection with EAEC and other enteric pathogens (Huang *et al.*, 2006a). EAEC infection may also contribute to malnutrition (Opintan *et al.*, 2010). Responsible for these effects of

Abbreviations: EAEC, enteroaggregative *Escherichia coli*; IL, interleukin; NTZ, nitazoxanide; RBD, regional basic diet; TNF, tumour necrosis factor.

A supplementary figure and a table are available with the online version of this paper.

EAEC may be multiple factors with putative virulence properties encoded by genes designated *aggR*, *aggA*, *aafA*, *aap* and *astA* (Shazberg *et al.*, 2003).

In a study of travellers' diarrhoea, Mohamed *et al.* (2007) found that diarrhoeagenic *E. coli* isolates were more often biofilm-producing strains than non-diarrhoeagenic strains. Biofilm production, mediated by aggregative adherence fimbriae, is a hallmark of the prototype EAEC strain 042 (Harrington *et al.*, 2005; Shamir *et al.*, 2010; Wakimoto *et al.*, 2004).

We have previously published data showing that infection can be elicited by oral challenge with the non-biofilm-producing EAEC strain JM221 and the biofilm-forming strain 042 in a neonatal mouse model (Roche *et al.*, 2010); however, there were important limitations to this model's usefulness. In the current study, we have described a weaned mouse model of EAEC infection, which we used to (i) identify a human-derived strain that is pathogenic in mice, (ii) describe an impressive impact of malnutrition on quantifiable end points (growth rates, stool shedding and tissue burden) and (iii) determine the preventive and therapeutic efficacy of subMIC concentrations of nitazoxanide (NTZ) on EAEC infection.

METHODS

Reagents. DNeasy, RNeasy and Stool DNA Extraction kits were all purchased from Qiagen. RNAlater was from Ambion. iScript cDNA Synthesis kits and SYBR Green Supermix were from Bio-Rad and the primers for PCR were purchased from Operon.

Mice. Male C57BL/6 mice, 21 days old on arrival, were purchased from Charles River Laboratories. Mice were maintained on 7 or 20% protein isocaloric diets from Research Diets (D09081701 and D09051102, respectively; Table S1, available in JMM Online) for the duration of the experiment. All animal studies were performed following approved guidelines of the University of Virginia Animal Care and Use Committee.

Preparation of EAEC for mouse challenge. EAEC strain 042 was obtained from Dr Nataro (University of Virginia, VA, USA). All bacteria were grown from glycerol stocks maintained at -80°C and prepared as described previously (Roche *et al.*, 2010). Briefly, 3 days prior to mouse challenge, EAEC strains JM221 or 042 were streaked onto Luria-Bertani agar plates. Cultures were grown for 48 h in a 37°C incubator until enlarged individual colonies were apparent. On the day of challenge, 14 colonies were picked from two plates of *E. coli* and grown in 20 ml Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose and phenol red in a 37°C shaking incubator. The cultures were monitored closely for 3–4 h until the medium started to turn from a pink to an orange hue (a pH change indicating a significant amount of metabolism of the culture medium). The cultures were analysed on a spectrophotometer by measuring optical density at 600 nm to assess growth. An OD_{600} of ~ 1.4 indicated optimal growth of $\sim 1.0 \times 10^9$ bacteria in 20 ml. Cultures were then spun down at 800 g for 5 min at 4°C . The supernatant was discarded and the bacterial pellet was resuspended in 2 ml fresh high-glucose DMEM, giving a c.f.u. ml^{-1} of $\sim 1.0 \times 10^{10}$. A 10 μl aliquot was diluted in TE buffer and 10^{-6} , 10^{-7} and 10^{-8} dilutions were plated on MacConkey agar and grown overnight in a 37°C incubator to assess the actual c.f.u. ml^{-1} of the inoculum. The bacteria were maintained on ice until administered to mice via oral

gavage using 22-gauge feeding needles at concentrations of $\sim 10^{10}$ in 100 μl high-glucose DMEM. Uninfected control animals were gavaged with 100 μl high-glucose DMEM as a vehicle/gavage control.

Animal studies. After challenging mice with EAEC as described above, the mice were weighed daily. Stools were collected every other day starting 24 h after challenge. Studies ranged from 14 to 35 days in duration. For intestinal burden analysis, some mice were euthanized on days 0, 4, 7 and 14. Tissues were collected from these mice, comprising duodenum, jejunum, ileum and colon, for DNA for real-time PCR to quantify EAEC. Weight at euthanasia was also recorded. Tissues were flash frozen in liquid nitrogen and stored at -80°C until the DNA was extracted.

Cytokine analysis. Total cellular RNA was obtained from each intestinal tissue using an RNeasy kit, and cDNA was synthesized from 1 μg RNA using iScript. For quantitative PCR analyses of cytokine mRNA abundance, the cDNA was diluted 1:8 and 4 μl of this dilution was used for each PCR. Reagents from the real-time PCR kit containing SYBR Green were used for quantitative PCR assays. The primer sequences used were as follows: β -actin, sense 5'-CCACCATGTACCCAGGCATT-3' and antisense 5'-CGGACTCATCGTACTCCTGC-3'; interleukin (IL)-4, sense 5'-AGGGACGCCATGCACGGAGA-3' and antisense 5'-TGCGAAGC-ACCTTGAAGCCC-3'; IL-12, sense 5'-CAAGGGCATGCTGGTGG-CCA-3' and antisense 5'-GCGGGTGTGAAGGCGTGAA-3'; IL-17, sense 5'-AGGGAGCCTGAGAGCTGCCC-3' and antisense 5'-AATCG-AGGCCACGCAGGTGC-3'; and tumour necrosis factor (TNF)- α , sense 5'-TGGCCAACGGCATGGATCTC-3' and antisense, 5'-AGCAAATCG-GCTGACGGTGT-3'.

The PCR conditions were: 95°C for 13 min, followed by 40 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 30 s, followed by melt-curve analysis. Data were analysed and are presented based on the relative expression method (Livak & Schmittgen, 2001). The formula used for the calculation was: relative expression = $2^{-(\Delta C_T - C_{T, \beta\text{-actin}})}$, where ΔC_T is the difference in C_T between the gene of interest (e.g. IL-4) and the housekeeping gene (β -actin). In this equation, S represents *E. coli*-challenged mice and C represents uninfected mice.

Alternatively, real-time PCR was used to measure the starting amount of nucleic acid by assaying each unknown cDNA on the same 96-well plate. For each gene, the results are presented as starting quantity of cDNA (pg) (μg tissue RNA) $^{-1}$.

Stool shedding and tissue burden. DNA was extracted from mouse faeces using a DNA Stool Mini kit for *E. coli* stool shedding analysis. DNA was extracted from mouse tissues using a DNeasy kit for determining *E. coli* tissue burden. Quantitative real-time PCR for AAP (the anti-aggregation protein dispersin), a protein specific to EAEC, was performed on the extracted DNA. An eight-point standard curve containing extracted DNA from 10^1 to 10^8 c.f.u. ml^{-1} was included in each PCR run, with an efficiency of 80–100%, yielding reliable detection down to 10^1 EAEC (10 mg stool or tissue) $^{-1}$. The *aap* gene (Sheikh *et al.*, 2002) was used as a specific target to quantify the presence of EAEC in stool and tissue samples. The primer sequences for *aap* were 5'-CTTGGGTATCAGCCTGAATG-3' (sense) and 5'-AACCATTTCGGTTAGAGCAC-3' (antisense). The PCR conditions were: 95°C for 13 min, followed by 40 cycles of 95°C for 30 s, 61.5°C for 30 s and 72°C for 30 s, followed by melt-curve analysis. Data were analysed and presented as c.f.u. (10 mg stool) $^{-1}$ or (10 mg tissue) $^{-1}$ based on comparison with the standard curve.

Data management and statistical analyses. Data for all experiments were recorded and analysed using GraphPad Prism software. Comparisons between groups were performed using one-way and two-way analysis of variance, as well as Mann-Whitney methods. Data are represented graphically as means $\pm$ SEM, in which each mean consisted of a minimum of three or four replicates (unless

noted otherwise in the figure legends). Comparisons between groups and tests of interactions were made assuming a two-factor analysis with the interaction term testing each main effect and with the residual error testing the interaction.

RESULTS

Diminished growth rate following EAEC challenge

Diarrhoea has been reported in humans infected with EAEC strains JM221 and 042 (Mathewson *et al.*, 1986; Nataro *et al.*, 1995). To explore whether either of these two human isolates of EAEC could induce overt disease in a small-animal model, 24-day-old nourished C57Bl/6 mice were administered $\sim 10^{10}$ c.f.u. by gavage with one of the two strains of EAEC (1.5×10^{10} c.f.u. JM221 or 1.0×10^{10} c.f.u. 042, as determined by plating on MacConkey agar; see Methods) or DMEM (vehicle control) via oral gavage. Shortfalls in weight gain were observed with each isolate, beginning 2–5 days post-challenge and continuing to day 14 (Fig. 1). Growth was significantly reduced ($\sim 15\%$) in mice given EAEC strain 042 compared with that of the uninfected controls (days 11–14; $P < 0.05$). These findings suggested that readily detectable and quantifiable disease consisting of growth rate reduction occurs in a weaned small-animal model when challenged with a human pathogenic strain of EAEC.

Persistent stool shedding following challenge with EAEC

Serial stools were collected and analysed for EAEC by quantitative real-time PCR. Challenge with either EAEC

isolate (JM221 or 042) revealed continued shedding of organisms at $>10^3$ c.f.u. $(10 \text{ mg stool})^{-1}$ over 2 weeks following challenge (Fig. 2). Although the original inoculum of EAEC strain JM221 was 50% greater than that of EAEC strain 042 in this experiment, both achieved a similar rate of shedding [$\sim 4.2 \times 10^4$ c.f.u. $(10 \text{ mg stool})^{-1}$] at 14 days post-challenge. In stools of control mice administered vehicle only (DMEM), no EAEC were detected. These findings suggested that a single inoculum of a human EAEC isolate induces disease and provides several quantifiable end points including growth shortfalls and an extended period of robust stool shedding in weaned, nourished C57Bl/6 mice. Based on these findings and those of a study demonstrating diarrhoea in human volunteers who ingested strain 042 but not in those who ingested strain JM221 (Nataro *et al.*, 1995), we elected to focus our subsequent studies on EAEC strain 042.

Impact of nutritional status on outcomes in EAEC-challenged mice

We have demonstrated previously that malnutrition enhances growth shortfalls and bacterial shedding in stools after EAEC challenge in a mouse neonatal model (Roche *et al.*, 2010). Whilst the studies above explored disease in nourished mice given a normal (20% protein) diet, we hypothesized that disease following EAEC challenge might be more overt in the setting of malnutrition, a condition common in children in developing countries who become infected with EAEC (Guerrant *et al.*, 2008). To test this, we chose to use a 'regional basic diet' (RBD; Table S1), designed to mimic the diet of undernourished children in north-eastern Brazil (Ueno *et al.*, 2011). Therefore,

Fig. 1. Growth rate of weaned, nourished mice challenged with strains of EAEC derived from humans. C57Bl/6 mice (24 days old) were administered one of the two strains of EAEC (JM221 or 042) or DMEM (vehicle control) via oral gavage at day 0. The mice were weighed daily to monitor growth rate. (a) EAEC strain JM221 (challenge inoculum 1.5×10^{10} EAEC per mouse; $n=5$ per group). ●, Uninfected; ◇, EAEC JM221. (b) EAEC strain 042 (challenge inoculum 1.0×10^{10} EAEC per mouse; $n=9$ per group). ●, Uninfected; □, EAEC 042. Shortfalls in growth were observed compared with the uninfected controls on days 11–14 ($*P < 0.05$). Similar results were observed in two independent studies with EAEC strains 042 and JM221.

Fig. 2. Stool shedding in weaned, nourished mice challenged with strains of EAEC. C57Bl/6 mice (24 days old) were challenged with one of two strains of EAEC (JM221 or 042) or with DMEM (vehicle control) via oral gavage at day 0. EAEC shedding continued for 2 weeks post-challenge. The inoculum used for each EAEC strain and the number of mice per group were the same as in Fig. 1. Similar results were observed in two independent studies.

beginning 3 days before EAEC challenge, weaned mice were fed either a standard 20% protein diet or the isocaloric RBD containing 7% protein and reduced fat and minerals (Ueno *et al.*, 2011), and were monitored for growth and stool shedding. A single inoculum of EAEC strain 042 by gavage was associated with growth shortfalls with both diets. Whereas a change in growth in challenged mice was noted as early as day 2–4 post-challenge, this became significantly different from that in uninfected mice by days 10 and 11 ($P < 0.05$, Fig. 3a). As expected, mice fed the RBD (malnourished) had significantly slower growth than those fed the control 20% protein diet. When stool shedding of EAEC was monitored after challenge, EAEC remained detectable up to day 14 (Fig. 3b). Moreover, RBD-fed mice, despite being given an identical inoculum as the nourished controls, shed consistently more EAEC beginning on day 3 ($P < 0.01$, days 3 and 12), suggesting that moderate malnutrition, engendered by a restriction in dietary protein, fat and minerals, is associated with more intense intra-luminal bacterial colonization, generally resulting in shedding at the level of $\geq 10^4$ c.f.u. (10 mg stool)⁻¹. Because of these findings, we incorporated moderate malnutrition with the RBD in subsequent studies, as we proceeded to examine the intestinal tissue burden of organisms and the associated level of secreted mucosal cytokines.

Nutritional status alters the distribution and tissue burden of EAEC

Although clinical disease in humans attributed to EAEC is regarded as occurring predominantly in the colon (Harrington *et al.*, 2006), we are unaware of *in vivo* studies

Fig. 3. Effect of diet on growth rate and stool shedding in weaned C57Bl/6 mice challenged with EAEC strain 042. C57Bl/6 mice (24 days old) were administered EAEC strain 042 (2.0×10^{10} per mouse) or DMEM (vehicle control) via oral gavage at day 0. (a) Growth rate following EAEC strain 042 challenge. * $P < 0.05$, days 10–13 for nourished EAEC-challenged mice compared with nourished uninfected controls; ** $P < 0.05$, days 11–14 for malnourished challenged mice compared with malnourished uninfected controls. RBD-fed mice had significantly lower growth rates than mice fed the control diet ($P < 0.01$, days 7–14 for malnourished uninfected versus nourished uninfected). ●, Nourished uninfected; ○, nourished EAEC 042; ▲, malnourished uninfected; △, malnourished EAEC 042. (b) Stool shedding following EAEC strain 042 challenge. * $P < 0.01$, days 3 and 12 for malnourished infected versus nourished infected ($n = 5$ mice per group). Similar results for growth rate and stool shedding were observed in two independent studies.

that have assessed other regions of the intestine for EAEC associated with mucosa, or of studies that have defined the impact of nutritional status on the distribution and quantity of mucosa-bound organisms. Therefore, nourished and malnourished mice were challenged by gavage with EAEC strain 042, euthanized on days 4, 7 and 14 post-challenge and used to provide stool-free tissue to assess colonization in the harvested duodenum, jejunum, ileum

and colon (Fig. 4). EAEC was found associated with colonic tissue in both nourished and malnourished infected mice on each day assessed (Fig. 4d). However, malnourished mice had a significantly greater EAEC tissue burden in the colon on day 4 than the nourished mice ($P<0.05$). Furthermore, in malnourished mice, two other regions of the intestine (duodenum and ileum) harboured $>10^{2.8}$ c.f.u. EAEC (10 mg tissue) $^{-1}$ after day 4 post-challenge, whereas in nourished infected mice, EAEC were not detected in these same regions of intestine ($P<0.0001$) (Fig. 4a, c). In particular, mucosal attachment of organisms persisted at $>10^{2.8}$ c.f.u. (10 mg tissue) $^{-1}$ for 14 days in the duodenum and for 7 days in the ileum of malnourished mice. These findings suggest a broader-than-expected capacity of EAEC to attach to and proliferate in major regions of the intestine in the malnourished host, and, furthermore, that this may be responsible for the greater and more prolonged stool shedding observed in the setting of moderate protein restriction (Fig. 3b).

Cytokine mRNA expression in intestinal tissues

To determine whether alterations in cytokines secreted locally in intestine may have accounted for the findings

above, cDNA was transcribed from RNA extracted from ileal tissue on days 1, 4, 7 and 14 after challenge with EAEC, and probed for mRNA specific for IL-4, IL-12, IL-17 and TNF- α (Fig. S1). Overall, mRNA levels for cytokines were highest on day 7, when growth shortfalls began to be noted, preceding the period (days 11–14) when failure to gain weight became significant (Fig. 3a). Thus, in the ileum of malnourished EAEC strain 042-challenged mice, day 7 elevations in IL-4 (12-fold) and IL-12 (fivefold) mRNA were found, whereas these cytokine mRNA levels were not increased in days 4 or 14. Expression of IL-17 and TNF- α followed a similar pattern with the greatest increase seen on day 7 post-infection. Furthermore, these day 7 elevations in cytokine mRNA correlated with decreased tissue burden of organisms in all four regions of intestine observed on day 7 (Fig. 4), suggesting that an inflammatory response is important for removal of the organism.

NTZ benefits mice challenged with EAEC strain 042

NTZ, a synthetic nitrothiazolyl-salicylamide derivative, is approved for the treatment of *Cryptosporidium* and *Giardia* infections in children, as well as for prevention of travellers'

Fig. 4. Tissue burden of EAEC in four regions of the intestine, as affected by nutritional status. C57Bl/6 weaned mice (24 days old) fed a control 20% protein diet (nourished) or RBD (malnourished) were challenged with 10^{10} c.f.u. per mouse of EAEC strain 042 via oral gavage. Four mice from each group were euthanized on days 4, 7 and 14 post-challenge, and tissue taken from the duodenum, jejunum, ileum and colon was analysed by quantitative PCR for the number of micro-organisms (10 mg tissue) $^{-1}$. Statistical significance is indicated by different letters: a versus b, $P<0.0001$; b versus c, $P<0.05$.

diarrhoea (Anderson & Curran, 2007). Furthermore, our group (Shamir *et al.*, 2010) demonstrated that NTZ prevents biofilm formation by EAEC at subinhibitory (i.e. subMIC) concentrations (Shamir *et al.*, 2010). To test the efficacy of NTZ for preventing disease in the EAEC mouse model used in this study, NTZ (0.4 or 2.0 mg per mouse) was administered by gavage for three consecutive days (days -1, 0 and +1 of EAEC challenge). Challenge with EAEC strain 042 (or DMEM in controls) consisted of a single inoculum given by gavage. Both doses of NTZ reduced the growth shortfalls seen with EAEC strain 042

infection ($P<0.05$, Fig. 5a). Stool shedding of EAEC strain 042 was significantly reduced in mice treated with the higher dose of NTZ (2.0 mg per mouse) but not with the lower dose of NTZ (0.4 mg per mouse), compared with infected DMEM-treated controls ($P<0.05$, Fig. 5b). When stool-free tissue was harvested from four regions of intestine on day 7 post-challenge and analysed for tissue burden of organisms by quantitative PCR, the number of EAEC in the duodenum and colon was significantly reduced by NTZ treatment ($P<0.05$, Fig. 5c). The EAEC burden in the jejunum was reduced by 75 %, whilst the

Fig. 5. Preventative effects of NTZ administration on growth rate, stool shedding and intestinal tissue burden in weaned, malnourished C57Bl/6 mice challenged with EAEC strain 042 (2.0×10^{10} per mouse) or with DMEM (vehicle control) via oral gavage at day 0. NTZ at 0.4 or 2.0 mg per mouse was administered on days -1, 0 and +1 of EAEC challenge. (a) Growth rates of challenged mice compared with uninfected controls. * $P<0.05$, days 11–13 ($n=10$ mice per group). ●, Uninfected; ○, EAEC 042; ▽, EAEC 042, NTZ 0.4 mg; □, EAEC 042, NTZ 2.0 mg. (b) Stool shedding of EAEC 042 in NTZ-treated and non-treated mice. * $P<0.05$, days 7, 9 and 11 comparing shedding in mice treated with the high dose of NTZ with uninfected control mice ($n=5$ per group). ○, EAEC 042; ▽, EAEC 042, NTZ 0.4 mg; □, EAEC 042, NTZ 2.0 mg. (c) Intestinal tissue burden of EAEC associated with epithelium in four regions of the intestine. Statistical significance is indicated by different letters: a versus b, $P<0.01$; c versus d, $P<0.05$ ($n=4$ mice per group). (d) Capacity of EAEC strains to produce biofilm *in vitro* as affected by the presence of NTZ at $25 \mu\text{g ml}^{-1}$. Statistical significance is indicated by different letters: a versus b, $P<0.01$.

EAEC burden in the ileum was higher, but neither result was statistically significant. An assay was performed to confirm the ability of 25 mg NTZ ml⁻¹ to inhibit biofilm formation by EAEC strain 042 ($P < 0.0001$, Fig. 5d). The non-biofilm-forming EAEC strain JM221 was included as a control. These results suggested a major benefit to the host of subMIC doses of NTZ on important quantifiable end points of growth, bacterial shedding in stool and regional tissue burden of organisms.

The benefit of NTZ administration after a disease-inducing EAEC challenge of the host remains unexamined. Therefore, malnourished 24-day-old C57Bl/6

mice were given, by gavage, one of two subMIC doses of NTZ or PBS on days 3, 4 and 5 following oral challenge with EAEC. Compared with the findings in infected but untreated controls, NTZ (2.0 mg per mouse) reduced growth shortfalls (days 7–14, $P < 0.05$, Fig. 6a). Whilst the effect on growth rate was positive, neither the number of organisms shed in stools nor the quantity of adherent organisms in four regions of the intestine was consistently reduced when NTZ was administered on days 3–5 after challenge (Fig. 6b, c). Overall, these findings demonstrated the benefit of NTZ in prevention and treatment of EAEC infections in a weaned, malnourished mouse model of EAEC infection.

Fig. 6. Effect of post-challenge NTZ on growth rate, stool shedding and intestinal tissue burden in weaned, malnourished C57BL/6 mice. Mice, NTZ doses and controls were the same as in Fig. 5, except that NTZ was administered on days 3, 4 and 5 after EAEC challenge (2.0×10^{10} c.f.u. per mouse). (a) Growth rates of mice after EAEC challenge on day 0. * $P < 0.001$, days 11–14, comparing NTZ at 2.0 mg per mouse versus infected controls; non-significant, days 1–14, comparing NTZ at 0.4 mg per mouse versus infected control ($n = 5$ per group). ●, Uninfected; ○, EAEC 042; ▽, EAEC 042 NTZ 0.4 mg; □, EAEC 042 NTZ 2.0 mg. (b) Stool shedding following EAEC challenge, shown as c.f.u. (10 mg stool)⁻¹ ($n = 5$ per group). ○, EAEC 042; ▽, EAEC 042 NTZ 0.4 mg; □, EAEC 042 NTZ 2.0 mg. (c) Intestinal tissue burden of EAEC in four regions of the intestine on day 7 following EAEC challenge.

DISCUSSION

EAEC has emerged as an increasingly important cause of diarrhoea worldwide. Watery diarrhoea caused by EAEC is often persistent and inflammatory (Nataro & Kaper, 1998). EAEC infections are associated with community outbreaks as well as travellers' diarrhoea, and are commonly reported in diarrhoeal disease occurring in human immunodeficiency virus-infected adults (Okeke & Nataro, 2001; Villaseca *et al.*, 2005). The specific pathogenesis and virulence factors responsible for EAEC-induced disease are not well understood; however, studies of components in clinical stool specimens and from *in vitro* model systems suggest that initiation of EAEC infection requires both specific adherence factors and production of putative toxin(s) (Vila *et al.*, 2000; Villaseca *et al.*, 2000).

The absence of animal models of EAEC infection has been a major obstacle in the elucidation of EAEC pathogenesis. Prior to our 2010 study (Roche *et al.*, 2010), only two animal models of EAEC infection had been reported: an intestinal loop model in rabbits and rats, and an infection model using 1-day-old piglets (Tzipori *et al.*, 1992; Vial *et al.*, 1988). Whilst these studies demonstrated the 'stacked-brick'-like adherence of EAEC to the intestinal epithelium, they did not evaluate longitudinal effects on the host; for example, the extent/duration of growth effects and persistence/intensity of shedding of the organism in stools. We recently demonstrated the ability to infect neonatal mice with EAEC, resulting in growth shortfalls and prolonged stool shedding (Roche *et al.*, 2010). Limitations of this previous model included the fragility of 4-day-old pups for *in vivo* studies and the small quantity of stool and tissue samples available for analysis from 4–6 g neonatal mice. In the current study, we compared two different EAEC strains, derived from humans, for their ability to infect nourished and malnourished, weaned 24-day-old C57Bl/6 mice. The effects of EAEC on the model were quantifiable and included changes in host growth rate, stool shedding and intestinal tissue burden of the micro-organism. Additionally, we demonstrated that *in vivo* infection could be prevented with NTZ, which we also found prevented biofilm formation by EAEC strain 042. This, together with the consistency of induction, presence of overt disease, convenience and ability to follow clinical parameters longitudinally following EAEC challenge, suggests that this new model will be useful for studies of both EAEC pathophysiology and treatment.

Published clinical data available on symptoms in patients associated with infection with specific strains of EAEC are limited but suggest heterogeneity among EAEC strains. For this investigation, we chose to study two strains: JM221, based on our previous findings in neonatal mice (Roche *et al.*, 2010), and strain 042, because it exhibits a putative virulence factor (biofilm production) lacking in JM221, and because a human volunteer study showed that three of five adults reported diarrhoea within 24 h following challenge with strain 042, whilst other strains did not elicit

symptoms (Nataro *et al.*, 1995). Using the weaned C57Bl/6 mouse, oral challenge with both strains was associated with growth shortfalls (Fig. 1) and persistent shedding of organisms over 14 days (Fig. 2). However, given the superior ability of EAEC strain 042 to cause growth shortfalls and its possession of a putative virulence factor (biofilm formation), we focused the remainder of the current studies on this strain.

Recurrent and persistent diarrhoea in young children in developing countries is associated with malnutrition as well as with the quality of the water supply and sanitation services. A vicious cycle ensues in which poor nutrition is associated with increased susceptibility to diarrhoea due to enteric pathogens, which in turn exacerbates malnutrition (Guerrant *et al.*, 2008). To model this, an RBD was developed for studies of malnutrition in mice that closely mimics the specific diet consumed in north-eastern Brazil (Teodósio *et al.*, 1990) where early weaning is associated with early onset of prolonged and subsequently persistent diarrhoea (McAuliffe *et al.*, 1986). This RBD had moderately reduced levels of protein, fat and micronutrients, and has been shown in mice to cause significant growth shortfalls compared with an isocaloric nutritious control diet (Ueno *et al.*, 2011). In the current study, EAEC-challenged C57Bl/6 mice administered the RBD for 3 days prior to a single oral challenge with EAEC strain 042 were observed to have a heightened level of disease compared with control mice fed an isocaloric control diet. Thus, malnourished mice had greater growth shortfalls (>8% weight difference by day 14; Fig. 3a), more shedding of EAEC in stools (>3 log₁₀ difference at days 7–14 post-challenge; Fig. 3b) and spread to additional regions of the intestine (duodenum, jejunum and ileum) that was not seen in nourished mice (Fig. 4). Growth shortfalls that were observed over 14 days following a single challenge with EAEC in the studies above indicated that there might be protracted intra-luminal colonization by this bacterium, which was indicated by the duration and intensity of stool shedding and the tissue burden of the organism. These findings demonstrated that malnutrition alone increased the severity of disease in C57Bl/6 weaned mice following a single oral challenge with EAEC.

NTZ is approved as a treatment for cryptosporidiosis and giardiasis in infants and small children as well as for the prevention of travellers' diarrhoea (Anderson & Curran, 2007). Although NTZ has recently been shown to prevent biofilm formation by EAEC *in vitro* (Shamir *et al.*, 2010), we know of no published studies testing the efficacy of NTZ in the prevention or treatment of EAEC infection in a vertebrate host. To test this, groups of weaned C57Bl/6 mice received by gavage one of two doses of NTZ on days –1, 0 and +1 of challenge with a disease-inducing inoculum of EAEC. Administration of 0.4 or 2.0 mg per mouse significantly reduced growth shortfalls by ≥10% of total body weight by day 1 post-challenge (Fig. 5a). NTZ at 2.0 mg per mouse also significantly reduced EAEC shedding in stools (Fig. 5b). Both NTZ doses significantly

reduced the number of EAEC micro-organisms associated with epithelium in the duodenum and colon 7 and 14 days after EAEC challenge (Fig. 5c). These data suggested that NTZ is of benefit in the prevention of EAEC infection in the weaned, malnourished mouse and may be worthy of trials in people exposed to EAEC. In subsequent studies of NTZ to test its efficacy as a therapy after exposure to EAEC has occurred, we administered NTZ on days 3, 4 and 5 following a single oral inoculum of EAEC. Whilst both doses were associated with a decrease in growth shortfalls, neither stool shedding nor tissue burden was reduced (Fig. 6a–c). The number of organisms was significantly increased in the ileum in the group administered 2.0 mg NTZ ($P < 0.001$). The benefit of subMIC amounts of NTZ may be due to disruption of biofilm formation *in vivo*. Additional studies should be carried out to examine the effect of NTZ on biofilm formation in the intestinal lumen.

Our study has several limitations that need to be addressed. First, although mice were malnourished to a moderate level (7% rather than a normal 20% protein diet) for 3 days prior to EAEC challenge, our model did not test more severe levels of malnutrition by incorporating weaning at an earlier time, malnourishing the dam, or both – circumstances that may more closely mimic subsets of children vulnerable to enteric infection in developing countries. Secondly, whilst softer stools were observed for several days after challenge with EAEC, overt diarrhoea with liquid stools was not seen. However, other studies have noted a significant increase in mucus secretion in response to EAEC infection (Harrington *et al.*, 2009). We plan to investigate these findings and include mucus quantification in our future studies. Thirdly, combinations of select media and conditions for EAEC propagation *in vitro* were not explored to assess their impact on potential virulence factors such as adherence fimbriae and toxin production by EAEC isolates. Furthermore, not assessed but able to be elucidated in the model are the effects of periods of malnourishment of different duration, multiple challenges or prolonged low-dose exposure to EAEC, and co-infections with other enteric pathogens – conditions likely to be experienced by people living in endemic areas. Lastly, using the current model, isolating lymphocytes for functional and phenotyping studies is difficult. These lymphocyte populations may be limited due to malnutrition and the young age of the mice.

In summary, EAEC strain 042 challenge of weaned, malnourished C57Bl/6 mice causing growth shortfalls and persistent stool shedding is a convenient, consistent model with quantifiable end points for studying the pathogenesis and potential therapeutics of EAEC infection. Malnutrition caused by feeding a diet with moderately lower protein, fat and micronutrients exacerbated growth shortfalls and stool shedding, as well as the level and distribution of EAEC tissue burden throughout the intestinal tract. Furthermore, NTZ prevented growth shortfalls in this model, whilst the higher dose administered prior to challenge significantly reduced pathogen stool shedding. Future studies may

address the aforementioned limitations of the current study as well as identifying which putative virulence factors associated with EAEC are responsible for disease in the model. In addition, this model may help elucidate potential mechanisms involved in the vicious cycle of enteric infection and malnutrition, as well as enabling the study of novel interventions to interrupt this cycle, which is so devastating to the development of children in impoverished areas worldwide.

ACKNOWLEDGEMENTS

This work was supported by the National Institute of Allergy and Infectious Diseases contract no. HHSN272201000056C to J.B.-R.

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