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Administration of capsule-selective endosialidase E minimizes upregulation of organ gene expression induced by experimental systemic infection with *Escherichia coli* K1

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Abstract

Many neurotropic strains of *Escherichia coli* cause potentially lethal bacteraemia and meningitis in newborn infants by virtue of their capacity to elaborate the protective polysialic acid (polySia) K1 capsule. Recombinant capsule depolymerase, endosialidase E (endoE), selectively removes polySia from the bacterial surface; when administered intraperitoneally to infected neonatal rats, the enzyme interrupts the transit of *E. coli* K1 from gut to brain via the blood circulation and prevents death from systemic infection. We now show that experimental *E. coli* K1 infection is accompanied by extensive modulation of host gene expression in the liver, spleen and brain tissues of neonatal rats. Bacterial invasion of the brain resulted in a threefold or greater upregulation of approximately 400 genes, a large number of which were associated with the induction of inflammation and the immune and stress responses: these included genes encoding C–X–C and C–C chemokines, lipocalins, cytokines, apolipoproteins and enzymes involved in the synthesis of low-molecular-mass inflammatory mediators. Administration of a single dose of endoE, 24 h after initiation of systemic infection, markedly reduced, but did not completely abrogate, these changes in gene expression, suggesting that attenuation of *E. coli* K1 virulence by removal of the polySia capsule may minimize the attendant inflammatory processes that contribute to poor outcome in these severe systemic infections.

INTRODUCTION

A wide range of encapsulated and capsule-free bacteria are able to gain entry to, and establish infection in, the blood compartment (Reacher *et al.*, 2000) but, in the absence of trauma or other forms of tissue injury, relatively few are able to invade the central nervous system (CNS) from the systemic circulation. Those that do invariably elaborate a polysaccharide capsule that provides protection from both humoral and cellular host defences (Tunkel & Scheld, 1993; Davidsen *et al.*, 2007). In neonatal bacterial meningitis (NBM), a leading cause worldwide of mortality and morbidity in newborn infants (Harvey *et al.*, 1999), up to 80 % of cases in industrialized countries are caused by just two

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Microarray data accession number: ArrayExpress E-MEXP-2406.

Five supplementary tables are available with the online version of this paper, listing primer pairs for amplification of genes examined using qRT-PCR for validation of microarray results, genes in neonatal rat liver and spleen altered in response to infection with *E. coli* K1, and genes upregulated more than threefold in neonatal rat liver and spleen following administration of endoE.

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encapsulated species: group B streptococci and *Escherichia coli* (Bonacorsi & Bingen, 2005; May *et al.*, 2005). Of the neuroinvasive *E. coli* isolates, 80–85 % express the K1 capsule (Robbins *et al.*, 1974), a homopolymer of α-2,8-linked polysialic acid (polySia) that mimics the molecular structure of the polySia modulator of neuronal plasticity in mammalian hosts (Rutishauser, 2008). In experimental *E. coli* K1 infection, the capacity to invade the CNS is critically dependent on polySia expression (Kim *et al.*, 1992) and the capsule contributes significantly to the overall virulence of K1 strains (Bortolussi *et al.*, 1978).

The continuing rise in the incidence of infections due to multi-drug-resistant bacteria has stimulated interest in the development of new antibacterial therapeutic paradigms based on the modulation of virulence: disarming pathogens by reducing the expression of virulence determinants essential for bacterial colonization, dissemination and survival within the host should enable immune defences to overwhelm the attenuated invader (Cegelski *et al.*, 2008; Taylor *et al.*, 2009). Studies from the pre-antibiotic era demonstrated that compromising the expression at the bacterial surface of the protective capsule facilitates rapid and effective resolution of potentially lethal bacterial infections (Avery & Dubos, 1931; Goodner *et al.*, 1932; Francis *et al.*, 1934). In similar fashion, we investigated the effect of enzyme-mediated selective removal of polySia on the course of infection in a model, adapted from Glode et al. (1977), that replicates many of the key features of the infection in the human neonate (Mushtaq *et al.*, 2004, 2005).

Two-day-old rats were fed *E. coli* K1 and the bacteria efficiently colonized the gastrointestinal (GI) tract within 24 h (Mushtaq *et al.*, 2004). The bacteria then gained entry to the blood compartment, where they expressed the non-*O*-acetylated form of polySia (Zelmer *et al.*, 2008); they subsequently accessed the CNS, probably through the blood–cerebrospinal fluid (CSF) barrier at the choroid plexus, a well-established portal for bacterial invasion of brain tissue (Nau & Brück, 2002). *E. coli* K1 could be localized in the meninges of rat pups using immunohistological techniques but, whilst the LPS O18 antigen at the bacterial surface could be readily visualized within brain sections at the neuronal cell–meningeal interface, no evidence was found for expression of *O*-acetylated or non-*O*-acetylated polySia in this protected niche (Zelmer *et al.*, 2008). Intraperitoneal (i.p.) administration of a polySia-specific capsule depolymerase (Tomlinson & Taylor, 1985; Leggate *et al.*, 2002) during the early phases of the infection prevented bacteraemia (Mushtaq *et al.*, 2004, 2005), meningeal invasion (Zelmer *et al.*, 2008) and death (Mushtaq *et al.*, 2004) from *E. coli* K1 infection, although colonization of, and persistence within, the GI tract was not interrupted (Mushtaq *et al.*, 2004). These studies, along with recent investigations of anti-anthrax therapies (Scorpio *et al.*, 2007, 2008), demonstrate the therapeutic efficacy of the ‘capsule-stripping’ approach.

In the K1 model, as in the natural infection in humans, the bacterial inoculum is acquired orally, susceptibility to infection is strongly age-dependent, bacterial invasion of the blood compartment is preceded by GI tract colonization, pathogenesis is dependent on expression of polySia at the bacterial surface and infection of the brain is limited to sites within the meninges, a distribution compatible with CNS entry via the choroid plexus and CSF (Tunkel & Scheld, 1993; Davidsen *et al.*, 2007). NBM is usually accompanied by extensive inflammation of the CNS and it is these local reactions, rather than the pathogen itself, that are largely responsible for the tissue damage associated with meningitis; indeed, progressive damage to the brain may accumulate after the site of infection has been rendered free of invading bacteria by chemotherapy (Täuber & Moser, 1999). Local induction of proinflamma-tory cytokines such as TNF-α, IL-1 and IL-6, a feature of bacterial meningitis, can be replicated by injection of LPS in models of the disease (Ramilo *et al.*, 1990; Täuber & Moser, 1999), and release of endotoxin following antibiotic therapy in infants with *E. coli* meningitis exacerbates the severity of the infection and increases mortality (Mustafa *et al.*,

1989). Failure of *E. coli* K1 to express polySia in the meninges will expose LPS as the outermost layer of the bacterial cell, resulting in a high probability of local cytokine and chemokine induction. The current study was designed to examine the degree of induction of a local transcriptional response to meningeal invasion by *E. coli* K1 and to determine the capacity of the polySia-specific capsule depolymerase endosialidase E (endoE) to prevent local inflammatory and tissue damage responses to the bacterial insult. The data indicate that interruption of the course of infection reduces, but does not completely abrogate, the local tissue response to bacterial invasion.

METHODS

Bacteria and capsule depolymerase

E. coli O18 : K1 strain A192PP was derived from *E. coli* A192, an isolate from a patient with septicaemia (Achtman *et al.*, 1983), by serial passage through neonatal rat pups as described previously (Mushtaq *et al.*, 2004; Zelmer *et al.*, 2008); the passaged strain was significantly more virulent in the rat pup model of infection than *E. coli* A192 (Mushtaq *et al.*, 2005). His_6-tagged endoE was produced in *E. coli* BL21(DE3) and purified by Ni-affinity chromatography as described previously (Mushtaq *et al.*, 2004). Recovered protein was pure as judged by SDS-PAGE, with no proteins detectable other than the 76 kDa recombinant endoE fusion product. Kinetic properties and stability of the recombinant enzyme are reported elsewhere (Leggate *et al.*, 2002; Mushtaq *et al.*, 2004). For i.p. administration, 20 μg doses of recombinant endoE for individual rat pups were formulated in 100 μl PBS.

Infection of neonatal rats and processing of tissues

Wistar rat litters (9–14 individuals), obtained from Harlan UK or bred in-house, were retained in a single cage with their natural mothers after birth. To establish infection by GI tract colonization, all members of a litter were fed 20 μl of mid-exponential-phase Mueller–Hinton (MH) broth culture of *E. coli* A192PP ($2–6\times10^6$ c.f.u. at 37 °C) from an Eppendorf micropipette (Mushtaq *et al.*, 2004). Intestinal colonization was assessed at 24 h intervals by MacConkey agar culture of perianal swabs; the presence of K1 in individually plated colonies was determined using phage K1E (Gross *et al.*, 1977) as described previously (Mushtaq *et al.*, 2004). Bacteraemia was detected by culture of blood samples taken from superficial veins in the footpad on MacConkey agar; K1 expression of individually plated colonies was detected with phage K1E. Animals were killed by decapitation and further blood samples taken at this stage for c.f.u. enumeration. Post-mortem, the brain, liver and spleen were excised aseptically, and each organ was cut into two pieces along the longitudinal axis of symmetry. One portion was transferred to 1 ml cold PBS and gently washed to remove blood; the washing process was repeated twice. The sample was then homogenized in 1 ml PBS using an Ultra-Turrax T8 homogenizer (IKA Werke). Homogenized aliquots were serially diluted and plated for bacterial enumeration; a selection of colonies was examined for K1 expression using phage K1E. The other portion was immediately placed in the RNA and tissue fixative RNAlater (Ambion) and maintained overnight at 4 °C; the tissue was then removed from the solution and stored at −70 °C prior to further processing. Control animals were fed 20 μl sterile MH broth and their organs and blood were processed in the same way as for K1-infected animals.

Serum cytokine assays

Serum was obtained from blood removed after cull of P4 rat pups and levels of IL-1β, IL-6, TNF-α and IL-10 were determined by ELISA assay, using appropriate antigen-affinity-purified anti-rat antibody pairs and detection reagents and rat recombinant cytokines as standards (capture antibodies: rabbit anti-rat in all cases; detection antibodies: biotinylated

rabbit anti-rat IL-1β, IL-10 and TNF-α, biotinylated goat anti-rat IL-6; Peprotech). All assays were performed at least three times and were highly reproducible.

Affymetrix GeneChip analysis

RNA was extracted from individual organs (liver, brain and spleen) using the Qiagen RNeasy Animal Tissue Midi kit. RNA integrity and concentration were determined with a Bioanalyser 2100 (Agilent) and a NanoDrop 1000 (Thermo Scientific). Labelled cDNA was hybridized to Affymetrix GeneChip Rat Genome 230 2.0 arrays (Affymetrix). The arrays comprised over 31 000 probe sets representing variants from more than 28 000 rat genes; there are 11 probe sets represented for each coding sequence (http://www.affymetrix.com). GeneChip expression analysis was performed using a sample extracted from uninfected rat pups of similar age to the reference control. Labelled cDNA synthesis, fragmentation, hybridization, washing and scanning of rat genome arrays were performed according to the Affymetrix GeneChip Expression Analysis Technical Manual (702232; Rev. 2). Hybridizations were incubated for 16 h at 45 °C. GeneChips were scanned using an Affymetrix Gene Array Scanner 3000. Hybridization data were analysed on GeneChip Operating Software (GCOS v1.4). DAT, CEL and CHP files were removed using the Data Transfer Tool (v1.1.1); CEL files were imported into the third-party data analysis software GeneSpring v7.3 (Agilent). GC-RMA normalization was performed on all data generated using a chip from a comparable uninfected rat pup as reference.

Quantitative RT-PCR (qRT-PCR)

RNA was extracted from brain, liver and spleen using the RNeasy Animal Tissue Midikit protocol (Qiagen) to ensure no DNA contamination. qRT-PCR was carried out using the Brilliant II SYBR Green QRT-PCR 1-Step kit (Stratagene). Briefly, 25 ng DNA-free RNA was used as template for each qRT-PCR in conjunction with gene-specific primer pairs (synthesized by Eurofins MWG Operon) designed using Clone Manager (Sci-Ed Software) for genes of interest to yield amplicons of 100–150 bp. The conditions for the qRT-PCR were: one cycle each of 50 °C for 30 s and 95 °C for 10 min, followed by 55 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Each reaction plate was duplicated and included ‘no template’, ‘no reverse transcriptase’, and ‘melting-curve analysis’ controls. All reactions were duplicated on each plate and performed using an Mx3000P instrument (Stratagene). The comparative threshold method was used to determine relative quantification of mRNA abundance and changes in mRNA expression level were calculated after normalization to the internal control gene, *actB*. The nucleotide sequences of the primers used in this study are presented in Supplementary Table S1, available with the online version of this paper.

RESULTS

Effect of single doses of endoE on progression of neonatal systemic infection

Feeding *E. coli* A192PP to 2-day-old (P2) rat pups led to GI tract colonization within 24 h (Fig. 1a). Bacterial translocation to the blood compartment began at P3–P4. By P7, the overwhelming majority of pups (90–100 %) yielded blood cultures that were positive for *E. coli* K1 and all bacteraemic animals had died by P9 (Fig. 1b). A requirement for polySia capsule expression during the early stages of infection was demonstrated by i.p. administration of endoE at P3; a single dose of the recombinant enzyme prevented death in 95–100 % of pups (Fig. 1b) and the animals remained healthy in spite of the fact that many displayed a transient bacteraemia (Fig. 1a). Bacterial blood counts were far higher and persisted for a longer period of time in untreated animals compared with those infected pups dosed with endoE (data not shown). EndoE had no effect on GI colonization by *E. coli* K1 (Fig. 1a).

Changes in organ gene expression following *E. coli* K1 infection

Pups were fed *E. coli* A192PP at P2; all litter members were infected in identical fashion and at the same time. Infected animals were culled at P4 and brain, liver and spleen removed for determination of bacterial burden and for RNA extraction. The rationale for choice of tissues was based on prior observations (Zelmer *et al.*, 2008): in our infection model, *E. coli* K1 traffics to the brain via the CSF and invades the meninges; bacteria associate with liver and spleen during the infection process and as major organs of the mononuclear phagocyte system they are likely to be involved in the removal of bacteria from the systemic circulation.

To evaluate the effect of severe systemic infection with *E. coli* K1 on organ gene expression and to ensure that RNA samples were obtained from the three different organs of animals at similar stages of the infectious process, three pups with blood counts in excess of 1×10^6 c.f.u. ml^{-1} were selected for further investigation (Table 1). As anticipated (Zelmer *et al.*, 2008), the bacterial burden in the brain, liver and spleen of these pups was substantial; for example, in two animals (41.7 and 41.8), counts from the brain tissue exceeded 10^9 and in the third pup (43.7) the burden was below 10^6. To gauge the extent to which brain involvement was accompanied by evidence of sepsis, blood levels of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α and the anti-inflammatory cytokine IL-10 were measured. Levels of IL-1β, IL-6, IL-10 and TNF-α were below the detection limit (0.06 ng ml^{-1}) in sham-treated control animals but high levels of the three pro-inflammatory cytokines and anti-inflammatory IL-10 were detected in serum from infected pups 41.7 (IL-1β, 3.8 ng ml^{-1}; IL-6, 2.1 ng ml^{-1}; IL10, 0.4 ng ml^{-1}; TNF-α, 15.0 ng ml^{-1}) and 41.8 (IL-1β, 2.3 ng ml^{-1}; IL-6, 4.1 ng ml^{-1}; IL10 0.8 ng ml^{-1}; TNF-α: 15.8 ng ml^{-1}). Insufficient serum was obtained from pup 43.7 to permit evaluation of systemic cytokine levels.

RNA extracted from brain, liver or spleen of the three severely infected animals was combined in equimolar amounts to create organ-specific pools and labelled cDNA samples derived from these pools were hybridized and compared with hybridized pooled sham-control labelled cDNA obtained from the corresponding organs of three uninfected pups. A strict threefold cut-off was applied in order to assess the more definitively differentially regulated genes once GeneChip Robust Multiarray Averaging (GCRMA) normalization had been performed. This is a greater stringency than used in some similar studies (Boyce *et al.*, 2002; Woodall *et al.*, 2005) and was applied to ensure that differentially expressed data were selected on the basis of biological relevance. Fully annotated microarray data from these experiments have been deposited in ArrayExpress (accession no. E-MEXP-2406): the gene expression from pooled brain tissue showed that 416 genes were upregulated threefold or more and 77 were downregulated at least threefold. The corresponding figures for pooled liver were 570 and 735, and for spleen 893 and 114 respectively. Modulated genes were spread across a wide range of functional categories but it was evident that a higher proportion of upregulated genes from brain tissue of infected rats were associated with inflammatory, immune and stress-induced phenomena, compared with the liver and spleen responses. In contrast to the liver response, modulation of gene expression in the brain was characterized by marked upregulation and modest downregulation (Table 2).

With both pooled liver and spleen arrays, upregulated infection-induced genes belonged to a wide range of functional categories, with genes involved in growth, differentiation, development, inflammation, the immune and stress responses, cell metabolism, transcriptional regulation and transport strongly represented (Table 2). Examples of genes that were strongly modulated are detailed in Supplementary Table S2 (liver) and Supplementary Table S3 (spleen). In liver, levels of gene expression of both C–X–C and C–C chemokines were markedly increased during infection, and genes involved in lipid metabolism, cholesterol and steroid biosynthesis and the innate immune response were also

prominently upregulated (Table 2, Supplementary Table S2). Evidence for a major reprogramming of liver metabolism in response to systemic *E. coli* K1 infection was further supported by extensive downregulation of genes in most functional categories. As a major lymphoid organ involved in blood filtration, the spleen responded to large numbers of K1 bacteria with a significant degree of gene upregulation. Very large increases in expression of genes encoding proteins involved in angiogenesis and tissue remodelling, such as fibrinogen, were evident (Supplementary Table S3), as were genes encoding proteins associated with the innate immune response. As with liver, spleen levels of C–X–C and C–C chemokines were increased (19.7-fold to 382.2-fold). Large increases in genes involved in splenic synthesis of complement components C9 (180.6-fold), factor I (54.4-fold), C3 (49.3-fold), factor H (39.5-fold) and C4-binding protein β (29.1-fold) contributed to a vigorous innate response, and a number of genes encoding apolipoproteins were also upregulated: *apoA-V* (66.4-fold), *apoA-I* (35.7-fold), *apoH* (35.4-fold), *apoCI* (20.0-fold), *apoCIII* (15.6-fold) and *apoE* (12.4-fold). Downregulation of genes in spleen samples was relatively modest (Table 2).

A high proportion of upregulated genes in pooled brain tissue from infected P4 pups was indicative of a vigorous local inflammatory response (Table 2). Some genes showed extremely large increases in the level of transcription following systemic infection and these were, in the main, associated with inflammatory and immune processes. Table 3 shows those genes that were upregulated to the greatest extent. Transcription of genes encoding C–X–C chemokine ligands 1, 2, 10 and 11 increased at least 200-fold, and *cxcl9* and *cxcl16* were overexpressed fourfold and fivefold respectively. Genes encoding C–C chemokine ligands 20 (751-fold), 2 (633-fold), and 5 (15-fold) were also highly upregulated, as were genes encoding complement components C3, C2, C1r, C1s and properdin. The full ArrayExpress gene lists indicated that a number of genes associated with protein ubiquitylation (Bhoj & Chen, 2009) were significantly upregulated. No clear pattern emerged from analysis of the relatively small number of genes that were downregulated after infection (Table 2).

Effect of endoE administration on organ gene expression

Intraperitoneal administration of a single 20 μg dose of endoE at P3, 24 h after the seeding of the K1 infection resulted in large reductions in bacterial organ counts at P4, in some cases completely eradicating the infection. Three P4 pups showing evidence of residual bacteria in blood, liver, spleen or brain following endoE administration (Table 1) were selected for investigation of organ gene expression. No detectable levels of cytokines IL-1β, IL-6, IL-10 and TNF-α, determined by ELISA, were found at P4 in infected pups treated with endoE.

Gene expression analysis showed that endoE treatment prevented infection-induced upregulation of the majority of genes in pooled liver samples: a total of only 32 genes, 19 of which possessed known functions, were overexpressed threefold or more. These included genes encoding pancreatitis-associated protein (*pap*; 28-fold) and orosomucoid 1 (7.2-fold), both characteristic of an acute-phase response, tryptophan hydroxylase (Tph; 84.2-fold), involved in serotonin synthesis, and lipocalin 2 (6.2-fold). The majority (19 of 32) were overexpressed less than fourfold. Similarly, only 18 splenic genes were upregulated in pooled endoE-treated samples: *tph* (13.6-fold) and *pap* (4.5-fold) were again evident. However, 735 genes from liver samples and genes from 1834 splenic samples, representing all categories listed in Table 2, were down-regulated in P3-treated P4 pups compared with uninfected sham controls, the large majority between three- and fivefold.

Although we were unable to obtain sufficient material to examine transcription in liver and spleen samples from individual animals, it was possible to obtain microarray data from brain tissue harvested from the three individual animals detailed in Table 1. There was some

variation between brain samples with respect to the numbers of genes up and downregulated threefold or more in individual rat pups within infected and infected treated groups (Fig. 2, Table 4). It is evident from the linear scatter plots shown in Fig. 2 that gene expression profiles of infected pups receiving an i.p. dose of endoE were significantly different from those of infected but untreated animals seeded with lethal numbers of *E. coli* K1. However, in infected, untreated P4 pups, genes that were over-expressed to the greatest extent were those found to dominate the pooled gene lists (Table 3 and ArrayExpress lists). Brain tissue from pup 43.7 showed upregulation of fewer genes than either pups 41.7 or 41.8, and down-regulation of a greater number than in these animals. Very few genes differentially regulated in brain samples were found to be even modestly (usually less than fivefold change) overexpressed in endoE-treated animals, in comparison with uninfected controls, and no obvious pattern was evident, although the gene encoding Map1b (microtubule-associated protein 1b) was upregulated in tissues from endoE-treated pups 45.2 and 45.4. Downregulated genes in both infected and treated animals belonged to a broad range of functional groups.

It was clearly important to determine if administration of endoE to healthy animals significantly affected gene expression. Therefore, 20 μg endoE was administered i.p. to three uninfected P3 pups, and the liver, spleen and brain were removed *post mortem* 24 h later. Tissues were processed as described above and gene expression profiles determined using pooled RNA from liver and spleen samples and RNA from brain tissue of individual pups. That endoE distributed to the systemic circulation from the peritoneal cavity was indicated by a threefold or greater upregulation of 21 genes in the liver and 36 genes in the spleen (see Supplementary Tables S4 and S5, respectively); a high proportion of the genes overexpressed in the spleen encoded protein-degrading enzymes (Supplementary Table S5). None of the genes in either liver or spleen were found to be upregulated in the corresponding tissues from infected (at P2), endoE-treated (at P3) pups when examined at P4 (ArrayExpress files). There was no upregulation of genes in brain samples from any of the three pups.

To confirm the validity of the microarray data, 11 genes (Table 5, Supplementary Table S1) were selected for qRT-PCR analysis; genes with varying degrees of modulation of expression were examined. Pearson correlation coefficient demonstrated a significant correlation (P=<0.0001) and an R^2 value of 0.9340, equivalent to a 93.40 % correlation, between the two methods of assessment of gene expression.

DISCUSSION

In human NBM, the release of bacterial wall components within the CNS stimulates a complex host response involving humoral and cellular immune mediators, reactive oxygen intermediates, matrix metallopeptidases, and other host-derived factors, leading eventually to functional and structural brain damage (Leib & Täuber, 1999; Yadav *et al.*, 2009). Cerebral white matter injury in neonates following *E. coli* K1 meningitis is particularly severe (Shah *et al.*, 2005) and is strongly influenced by poorly defined host genetic factors (Lyons *et al.*, 2009). Local inflammation results initially from binding of LPS and other bacterial products to CD14 molecules on monocytes and macrophages, leading to synthesis of a large number of cytokines and chemokines that drive an innate response that often fails to control or eliminate the pathogen (Täuber & Moser, 1999). The proinflammatory cytokines IL-1β, IL-6 and TNF-α are present in CSF during meningitis and are produced locally (Møller *et al.*, 1991; Täuber & Moser, 1999); the concentrations of these effectors in serum are usually only elevated in meningitis when patients display signs of accompanying sepsis (Täuber & Moser, 1999). In addition, C–X–C and C–C chemokines often feature prominently in the CSF of bacterial meningitis patients (Inaba *et al.*, 1997; Spanaus *et al.*,

1997), as do the anti-inflammatory cytokines IL-10 and TGF-β, indicating the complexity of the host response to meningitis pathogens.

It is clear from the present study that the *E. coli* K1 neonatal rat model that we employ replicates in broad terms the local tissue inflammatory response associated with bacterial meningitis in humans (Täuber & Moser, 1999; Polin & Harris, 2001), at least in animals with a very high blood and brain bioburden. In our model, all members of a litter are infected concomitantly but the time of initiation and the extent of bacteraemia and tissue involvement vary over the course of 2–5 days post-infection (Mushtaq *et al.*, 2004) and for this reason we selected for study those animals showing extensive bacteraemia at P4, rather than pooled tissues from all animals in an infected litter. It is highly likely that the selected animals were close to death and are examples of overwhelming infection that may not be typical of many examples of the human disease, but they provide an opportunity to determine the potential degree of host gene modulation during overt, late-stage infection. The profound changes in gene expression in the liver and spleen, featuring a wide range of host functions, are likely to represent an attempt to enrich the supply of immune mediators, such as various complement components and cell adhesion molecules, to defend the organ against stress-induced damage and to clear the invading bacteria. We provide strong evidence that these organs also mount a local inflammatory response.

A different pattern of infection-induced gene expression was detected in the brain, which is the primary target organ for *E. coli* K1 in neonatal infection (Polin & Harris, 2001), as evidenced by the particularly large number (400–500) of genes differentially regulated, most of which were upregulated. The rodent brain is very active with respect to gene transcription, with about 80 % of all genes concomitantly expressed in highly localized fashion (Lein *et al.*, 2007), and this infection-induced upregulation was therefore super-imposed on an already highly active organ. Around 36 % of upregulated genes that could be assigned to functional categories were associated with inflammatory, immune and stress responses and a high degree of modulation of those cytokines and chemokines indicative of local tissue responses in the human neonate (Täuber & Moser, 1999) was evident; also prominent were genes encoding proteins involved in serotonin and prostaglandin synthesis, lipid transport and catabolism, complement activation, tissue remodelling and lipocalin synthesis (Table 3); the latter family of proteins are associated with regulation of the inflammatory response to LPS-mediated endotoxaemia (Sunil *et al.*, 2007). In two of the three infected pups, we found evidence of systemic levels of key cytokines, suggesting a threshold level of sepsis related to the bacterial burden in the blood circulation. Fewer genes were over-expressed in brain tissue from pup 43.7 (Table 4), which carried a lower bioburden than either pup 41.7 or pup 41.8, but the ArrayExpress data files clearly show that the prominent, highly upregulated genes (some of which are shown in Table 3) feature prominently in microarray data from all three pups. In the light of these responses we are planning further, more extensive studies of the transcriptional response in animals with less severe infection. We have previously shown that, following penetration of the meninges by *E. coli* K1 strain A192PP, the polySia capsule does not appear to be expressed at the bacterial surface (Zelmer *et al.*, 2008). It is likely that unmasking of the LPS-containing outer membrane exacerbates the local inflammatory response and we intend to determine if sites of synthesis of immune mediators coincide with bacterial foci of meningeal infection.

Conventional antibiotic chemotherapy in neonatal meningitis generally involves combinations of broad-spectrum antibiotics, usually including an aminoglycoside and a third-generation cephalosporin, administered for 2 or 3 weeks (Polin & Harris, 2001; Fernando *et al.*, 2008). Such regimens are likely to engender the release of cell wall components, in particular LPS, which will continue to drive local inflammation despite the high likelihood that use of these antibiotics will eradicate the infectious agent; residual

bacterial components almost certainly contribute to ongoing damage to the CNS in the absence of the pathogen in these infections (Täuber & Moser, 1999). One potential benefit of the 'capsule-stripping' approach is that enzyme-mediated attenuation is likely to prepare the K1 bacteria for uptake and disposal by phagocytic cells of the immune system (Mushtaq *et al.*, 2005) by processes that do not involve bacterial lysis and subsequent release of proinflammatory mediators. The current study provides some evidence in favour of this scenario, as dosing with endoE abrogates to a very large degree the overexpression of host genes in the brain (Fig. 2), although it is highly probable that polySia is removed from the *E. coli* surface during the blood phase of infection and relatively few bacteria transit to the CNS in comparison to the situation in infected but untreated pups. Virtually all endoE-treated animals remained healthy, displaying no symptoms of infection, even though low numbers of bacteria could be cultured from the major organs. There was clear evidence (Table 4), in spite of a successful therapeutic outcome following endoE administration, of some reprogramming of gene transcription in the brain, but this did not appear to involve significant upregulation of the local immune response. Interestingly, although administration of endoE to healthy P3 pups did not result in any upregulation of gene expression in the brain, a small number of genes were overexpressed in liver and spleen, suggesting that the enzyme gained access to the blood compartment in these uninfected animals and was then removed from the circulation by the major organs of the monocyte phagocytic system, as evidenced by increased rates of transcription of protein-degrading enzymes, particularly in the spleen. The liver and spleen genes upregulated by endoE did not appear to be modulated in infected pups treated with endoE. 'Capsule-stripping' remains, therefore, a therapeutic approach with the potential to prevent the induction of processes that contribute to the significant mortality and morbidity associated with this severe systemic infection (Galiza & Heath, 2009).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CNS	central nervous system
CSF	cerebrospinal fluid
endoE	endosialidase E
GC-RMA	GeneChip robust multiarray averaging
GI	gastrointestinal
i.p.	intraperitoneal
NBM	neonatal bacterial meningitis
polySia	polysialic acid
qRT-PCR	quantitative RT-PCR

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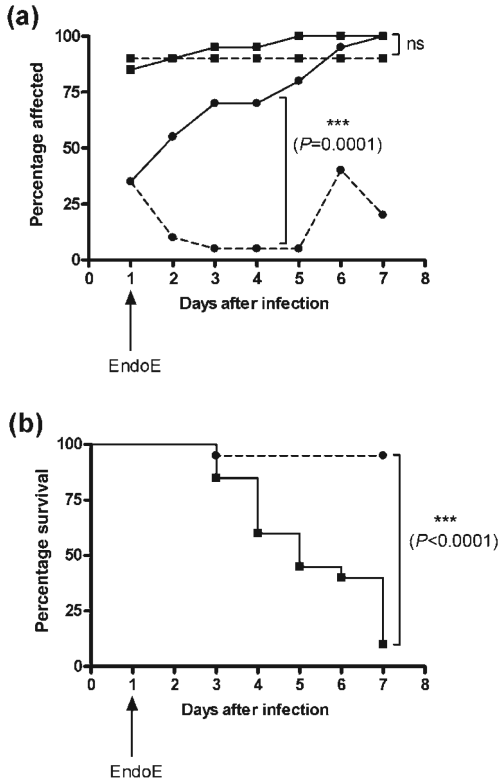

Fig. 1.

Outcome of feeding *E. coli* K1 strain A192PP to 2-day-old (P2) rats. Animals were left untreated or 20 μg endoE was administered at P3; n=20 in each group. (a) Colonization and bacteraemia were determined daily over P3–P9, although *E. coli* expressing K1 were detected in perianal swabs from almost all animals at P3. For purposes of statistical analysis using the GraphPad Prism logrank test, pups were scored as bacteraemic if one or more daily blood samples yielded K1-positive colonies, even if no bacteraemia was detected at later time points. The difference regarding bacteraemia between the treated and control group was highly significant (P=0.0001), whilst colonization was not significantly different between the two groups (ns; P=0.4309). ■–■, Colonization control; ■- -■, colonization treated; ●–●, bactaeremia control; ●--●, bacteraemia treated. (b) Deaths were recorded over the period represented by P3–P9. Survival curves of treated and control animals are significantly different (P<0.0001). ■–■, Survival control; ●- -●, survival treated.

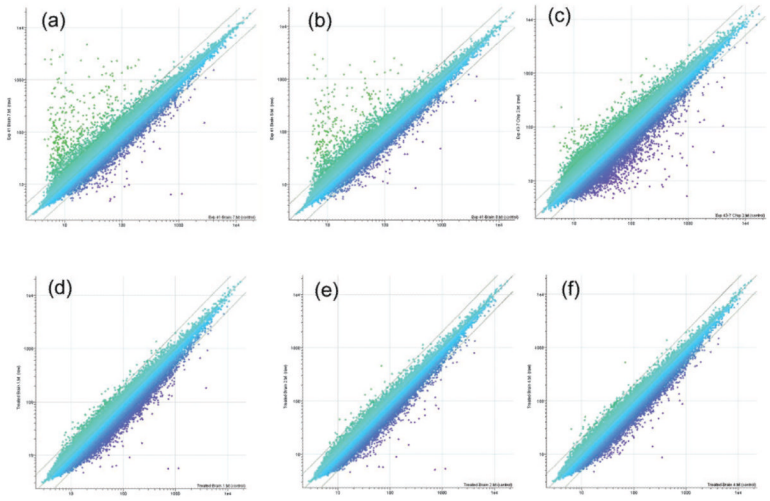

Fig. 2.

Modulation of gene transcription in the brain of individual rat pups 48 h after infection with *E. coli* A192PP (a–c) and following i.p. administration of endoE to infected animals (d–f). Rats were fed bacteria at P2 and culled at P4; endoE (20 μg) was dosed at P3. These linear scatter plots compare expression of individual genes from infected or infected/treated animals (*y*-axis) with uninfected P4 pups fed sterile MH broth at P2 (*x*-axis). Parallel lines indicate twofold increase (green points) or decrease (blue points) of gene transcription. Data were collated within GeneSpring v7.3, and genes up and downregulated threefold were evaluated by Student's *t*-test ($P<0.05$). (a) Pup 41.7; (b) pup 41.8; (c) pup 43.7; (d) pup 45.1; (e) pup 45.2; (f) pup 45.4.

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Table 1

Viable bacteria in blood and organs from *E. coli* A192PP-infected neonatal rat pups

Pups were fed 20 μl of *E. coli* A192PP culture 2 days after birth (P2), and blood and organs were harvested 48 h later (P4). For endoE treatment, the enzyme was administered i.p. to A192PP-fed pups at P3, and blood and organs were harvested at P4.

Pup Intervention	Brain*	Liver*	Spleen*	Blood†
41.7None	24.45×10^{8}	12.20×10^{6}	31.75×10^{4}	$>1\times10^{6}$
41.8None	12.28×10^{8}	31.05×10^{7}	25.07×10^{6}	$>1\times10^{6}$
43.7None	57.5×10^{4}	17.60×10^{6}	16.28×10^{6}	$>1\times10^{6}$
45.120 μg endoE	36	104	44	40
45.220 μg endoE	8	556	68	880
45.420 μg endoE	0	4	0	0

*Total c.f.u. per organ.

†C.f.u. per ml blood.

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Table 2

Functional categorization of genes expressed in neonatal liver, spleen and brain altered at least threefold following systemic infection with *E. coli* K1

The number of genes in each category is shown. Genes were assigned manually to functional categories as broadly defined by Moen *et al.* (2008); multifunctional proteins were assigned based on their primary role. Arrays were performed using pooled cDNA from three infected rats and compared with uninfected control animals.

Functional category	Liver		Spleen		Brain	
	Up	Down	Up	Down	Up	Down
Adhesion and migration	18	14	26	4	10	2
Angiogenesis	5	1	9		1	
Apoptosis	16	3	10	1	6	2
Coagulation	5	1	6		3	1
Cytoskeletal reorganization	14	3	5	1	1	
DNA repair		5	2		5	
Electron transport	18	40	27	1	6	
Exocytosis/endocytosis	1	3	3	1	1	
Growth, differentiation and development	34	62	88	5	32	4
Inflammation, immune and stress response	62	20	98	7	114	
Integral membrane proteins *	9	6	7	2	2	1
Iron homeostasis	2					
Metabolism: biosynthesis and catabolism	74	179	167	31	36	6
Nuclear structure	10	5	9			
Protein and RNA processing	19	49	74	4	39	5
Signal transduction	27	29	33	1	11	5
Transcriptional regulation	30	23	35	6	24	4
Transport	37	53	54	8	22	7
No. of genes affected	570	735	893	114	416	77
No. of genes categorized †	381	496	653	72	313	37

* Genes encoding molecules with features of transmembrane proteins but of no known function were assigned to this group.

† Genes encoding non-membrane proteins with no known function according to GeneSpring database were excluded from this analysis.

Table 3

Highly upregulated genes in neonatal rat brain following infection with *E. coli* K1

The 37 most highly upregulated genes of known function in pooled brain tissue from three infected pups are listed.

Gene	Gene name	Function	Fold change
Cxcl1	C–X–C chemokine ligand 1	Inflammatory response; chemotaxis	2229
Cxcl2	C–X–C chemokine ligand 2	Inflammatory response; chemotaxis	1668
Tph	Tryptophan hydroxylase	Serotonin biosynthesis	1419
Csf3	Colony stimulating factor 3	Immune response	767.8
Ccl20	C–C chemokine ligand 20	Inflammatory response; chemotaxis	751.5
Cxcl11	C–X–C chemokine ligand 11	Immune response	664.8
Ccl2	C–C chemokine ligand 2	Inflammatory response; chemotaxis	633.0
Il1b	Interleukin 1β	Inflammatory response	379.8
Il6	Interleukin 6	Acute phase/immune response	339.8
Mmp13	Matrix metallopeptidase 13	Proteolysis; collagen metabolism	302.2
Lcn2	Lipocalin 2	Transport/apoptosis	288.2
Cxcl10	C–X–C chemokine ligand 10	Inflammatory response; chemotaxis	286.7
Ptges	Prostaglandin E synthase	Prostaglandin synthesis; humoral response	182.1
Il1a	Interleukin 1α	Inflammatory response	181.0
Mmp9	Matrix metallopeptidase 9	Proteolysis; collagen metabolism	116.0
Plek	Pleckstrin	intracellular signalling cascade	99.4
Nos2	Nitric oxide synthase 2	NO synthesis; defence response to bacteria	85.9
Pglyrp1	Peptidoglycan recognition protein 1	Immune/defence response to bacteria	69.1
C3	Complement C3	Immune response: complement activation	68.7
Ptges	Prostaglandin E synthase	Prostaglandin synthesis; humoral response	62.1
Plscr1	Phospholipid scramblase 1	Immune response; phospholipid transport	62.0
Cebpd	CCAAT/enhancer binding protein δ	DNA-binding regulator of transcription	61.0
Sele	Selectin (endothelial)	Inflammatory/defence response	58.6
Serpine 1	Serine/cysteine proteinase inhibitor	Blood coagulation; regulation angiogenesis	57.7
S100a8	Calgranulin A	Acute inflammatory response; Ca^{2+} binder	57.3
Cd14	CD14 antigen	Inflammatory/immune response	56.5
Pla2g2a	Phospholipase A2, group IIA	Lipid catabolism (platelets, synovial fluid)	54.7
Ptgs2	Prostaglandin-endoperoxide synthase 2	Prostaglandin synthesis	53.6
Serpinb2	Serine/cysteine proteinase inhibitor	Wound healing	53.6
Ccl4	Small inducible cytokine A4	Inflammatory response; chemotaxis	53.0
Il1rn	Interleukin receptor antagonist	Lipid metabolism; immune response	50.2
Bf	B-factor, properdin	Complement activation, alternative pathway	48.4
Plaur	Plasminogen activator, urokinase receptor	Blood coagulation; signal transduction	42.8
Cp	Ceruloplasmin	Copper/iron ion homeostasis	40.6
Icam1	Intercellular adhesion molecule 1	Cell–cell adhesion; immune cell migration	37.2
Timp1	Inhibitor of metalloproteinase 1	C21-steroid hormone biosynthesis	36.1

Gene	Gene name	Function	Fold change
Selp	Selectin (platelet)	Inflammatory response; cell adhesion	34.1

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Table 4

Up- and downregulated genes in brain tissue from individual rat pups infected with *E. coli* K1

Pup	Intervention	Up*	Down*
41.7	None	403	77
41.8	None	299	60
43.7	None	157	560
45.1	20 μg endoE	11	159
45.2	20 μg endoE	7	92
45.4	20 μg endoE	6	94

*Number of genes modulated threefold or more at P4, compared with pooled brain tissue from sham-treated control P4 pups.

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Table 5

Comparison of fold-change in gene expression, as determined by microarray and qRT-PCR, in 11 genes upregulated in neonatal rats infected with *E. coli* A192PP; qRT-PCR was performed using Brilliant II SYBR Green chemistry and produced a significant correlation (P=<0.0001, R^2=0.9340) with microarray results as determined by Pearson correlation coefficient analysis

Gene symbol	Fold-change	
	Microarray	qRT-PCR
Ffg	706.4	625.92
Spin2c	625.6	683.25
A2m	582.9	434.95
Ccl2	111.9	149.92
Lcn2	85.21	215.92
Pglyrp1	30.48	89.24
Cp	27.95	4.02
Ptgs2	23.97	5.69
Icam1	20.01	15.27
RGD:621261	16.62	21.97
Cebpd	15.07	47.93