Escherichia coli strains colonising the gastrointestinal tract protect germfree mice against *Salmonella typhimurium* infection

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Abstract

Background—*Escherichia coli* is part of the normal gastrointestinal microflora which exerts a barrier effect against enteropathogens. Several *E coli* strains develop a protective effect against other Enterobacteriaceae.

Aims—Two *E coli* strains, EM0, a human faecal strain, and JM105 K-12 were tested for their ability to prevent in vivo and in vitro infection by *Salmonella typhimurium* C5.

Methods—Inhibition of C5 cell invasion by *E coli* was investigated in vitro using Caco-2/TC7 cells. The protective effect of *E coli* was examined in vivo in germfree or conventional C3H/He/Oujco mice orally infected by the lethal strain C5.

Results—EMO expresses haemolysin and cytotoxic necrotising factor in vitro. In vitro, the two strains did not prevent the growth of C5 by secreted microcins or modified cell invasion of C5. In vivo, establishment of EM0 or JM105 in the gut of germfree mice resulted in a significant increase in the number of surviving mice: 11/12 and 9/12, respectively, at 58 days after infection (2×10^6 /mouse) versus 0/12 in control germfree group at 13 days after infection. Colonisation level and translocation rate of C5 were significantly reduced during the three days after infection. In contrast, no reduction in faecal C5 excretion was observed in C5 infected conventional mice (1×10^8 /mouse) receiving the EM0 or JM105 cultures daily.

Conclusions—Establishment of *E coli* strains, which do not display antimicrobial activity, protects germfree mice against infection and delays the establishment of C5 in the gut. Possible mechanisms of defence are discussed.

(Gut 2001;49:47–55)

Keywords: *Escherichia coli*; gastrointestinal infection; *Salmonella*; germfree mice; bacterial antagonism

The normal resident intestinal microflora is the major factor protecting animals and humans against intestinal colonisation by pathogenic bacteria.^{1–2} In rare cases it has been possible to isolate the bacterial species responsible for protection and to elucidate the mechanism of protection. *Escherichia coli* is one of the first bacterial genera, along with *Streptococcus*, to colonise the intestine of human^{3–6} and animal newborns, including mice, rats, piglets, and

chickens. It has been reported that several *E coli* strains develop a protective effect against antibiotic resistant, colicin sensitive, and enterotoxigenic *E coli*.^{7–13} Barrow and Tucker¹⁴ demonstrated strong inhibition of intestinal colonisation of chicken caecum with *Salmonella typhimurium* by pretreatment (24 hours before) with strains of three *E coli* strains but the strains were not equally effective against other strains of *Salmonella*.

Here we examined the efficacy of an *E coli* K-12 JM105 strain and a human faecal *E coli* strain, EM0, against *S typhimurium* infection in mice and in vitro in human cultured cells. *Salmonella* can be divided in two groups—those that typically produce typhoid-like infections in humans or animals and those that produce non-life threatening gastroenteritis (food poisoning). The severity of the disease depends on the serovar and the host. In nearly all *Salmonella* infections, the bacteria multiply in the lumen in the small intestine, rapidly penetrate the intestinal mucosa, and reach the mesenteric lymph nodes (MLN) where they multiply. Most infections do not proceed beyond the local lymph nodes. Some more invasive strains can spread into the circulatory system and to deeper tissue such as the spleen and the liver. Infected bile causes a secondary intestinal infection and leads to the carrier state.¹⁵ In mice, *S typhimurium* induces typhoid-like disease and its primary site of invasion is the terminal ileum.^{16–17} Peyer's patches and M cells of the small intestine are the first to be invaded¹⁸ allowing the *Salmonella* to reach the MLN, and then the spleen and the liver.¹⁶ It is known that mouse genotype plays a critical role in the sensitivity to the lethal effect in systemic salmonellosis induced by *S typhimurium*. We have chosen C3H/He/Oujco mice which have the same characteristics as C3H/He mice. This breed¹⁹ is resistant to *S typhimurium* C5 given subcutaneously ($LD_{50} > 10^5$). We observed that germfree C3H/He/Oujco mice died in approximately eight days after oral infection by 2×10^6 bacteria/mouse whereas their conventional counterparts survived.²⁰

The *E coli* strain EM0 was originally isolated from the faecal flora of a healthy human volunteer.⁹ It has been shown to have a protective

Abbreviations used in this paper: LB broth, Luria-Bertoni broth; hlyA, α -haemolysin; cdt, cytolethal distending toxin; PBS, phosphate buffered saline; TSA, tryptic soy agar; SS agar, *Shigella* and *Salmonella* agar; PCR, polymerase chain reaction; LDH, lactate dehydrogenase; CNF, cytotoxic necrotising factor; MLN, mesenteric lymph nodes; IL, interleukin; TNF- α , tumour necrosis factor α .

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Accepted for publication
19 December 2000

effect against establishment of *E. coli* antibiotic resistant strains in germfree mice,⁹ against antibiotic resistant enterobacteria in human newborns,^{13, 21} and gnotobiotic mice.²² It has a protective effect against ETEC K88 in piglets.^{9, 12} The mechanism suggested is either “adaptation” of the EM0 strain inoculated first to germfree mice as ultrastructural differences in cell morphology were observed in vivo but disappeared in vitro cultures,⁹ or a slower generation time for the resistant enterobacteria mutant.²² Moreover, this strain plays a role in the development of the immune system in germ-free mice (MC Moreau, personal communication), inducing oral tolerance to ovalbumine²³ and the production of cytokines in peritoneal and bone marrow macrophages.^{24, 25}

To investigate the antagonistic activity of the *E. coli* strains against *S. typhimurium* oral infection in vivo, two experimental designs were used: *E. coli* were given to either: (i) germfree animals to study the effect of the extensive gut colonisation by *E. coli* on protection against *S. typhimurium* infection; or (ii) conventional C3H/He/OuJco mice to examine if an *E. coli* strain, given daily as a probiotic that can transit along the gut, exerts an antagonistic effect in situ against infecting *S. typhimurium*. In vitro, *S. typhimurium* invades enterocyte-like Caco-2 cells.²⁶ We next examined if the *E. coli* strains exerted an antibacterial effect in vitro—that is, whether they could inhibit invasion of cultured human enterocyte-like Caco-2/TC7 cells by *S. typhimurium*.

Methods

BACTERIAL STRAINS AND GROWTH CONDITIONS

The *E. coli* strain EM0 was originally isolated from the faecal flora of a healthy human volunteer⁹ and was kindly supplied by Y Duval (INRA, Jouy-en-Josas, France). The strain JM105²⁷ is an *E. coli* K-12 strain²⁸ kindly provided by M Fons (INRA). *S. typhimurium* strain C5 was kindly provided by M Popoff (Institut Pasteur, Service des Entérobactéries, Paris, France).²⁹ *E. coli* strains and *S. typhimurium* C5 were grown in Luria-Bertoni broth (LB) (Difco, Detroit, Michigan, USA) for 18 hours at 37°C under aerobic conditions. After centrifugation, the bacteria were resuspended and adjusted to the appropriate concentration in sterile phosphate buffered saline (PBS) for assay. Viable counts of *E. coli* or *S. typhimurium* were obtained after plating suitable dilutions on tryptic soy agar (TSA) (Difco) and incubation at 37°C for 18 hours. When differential counts of *S. typhimurium* from *E. coli* were necessary, viable bacteria were enumerated on *Shigella* and *Salmonella* agar (SS agar) (Difco). After incubation for 24–48 hours at 37°C, colonies of *S. typhimurium* were black and easily distinguishable from *E. coli* colonies or normal resident enterobacteria which gave red or pink colonies.

PCR

Polymerase chain reaction (PCR) detection of α -haemolysin (*hlyA*), cytotoxic necrotising factor 1 and 2 (*cnf 1-2*), and cytolethal distending toxin (*cdt*) sequences were performed with gene

Amp PCR system 2400 (Perkin-Elmer Applied Biosystems, Courtabeuf, France). Primers *hlyA1* (5'-CTC ATT GGC CTC ACC GAA CGG-3') and *hlyA2* (5'-GCT GGC AGC TGT GTC CAC GAG-3') were designed to amplify a 299 bp internal fragment from *hlyA* gene. Primers *cnfA* (5'-CTG AGC GGC ATC TAC TAT GAA G-3') and *cnfB* (5'-CCT GTC AAC CAC AGC CAG TAC-3') were designed to amplify a 626 bp internal fragment from *cnf* gene. Primers *cdt1* (5'-GTW GCR ACY TGG AAY YTK CAR GG -3') and *cdt2* (5'-KCM GGY KMR CGR TTR AAA TCW CC -3') were designed to amplify a 500 bp internal fragment from *cdt* gene. Colony PCR was carried out using “PCR beads ready to go” (Amersham Pharmacia, Saclay, France) according to the manufacturer's protocol. After initial denaturation (five minutes at 94°C), the samples were subjected to 30 cycles of amplification, each of which consisted of the following steps: 30 seconds at 94°C, 30 seconds at 57°C, and one minutes at 72°C. A final extension step of 10 minutes at 72°C was conducted. PCR products were examined on 1% agarose gels. As positive controls, we used *E. coli* SE124 *hly*⁺,³⁰ *E. coli* J96 *hly*⁺, and *cnf*⁺,³¹ kindly provided by P Boquet (Inserm U452, Nice, France), and *E. coli* DH5apOME01,³² kindly provided by E Oswald (Ecole Nationale Vétérinaire, Toulouse, France).

HAEMOLYSIN ASSAY

For qualitative evaluation of haemolysin production, isolates were inoculated onto Columbia agar plates (Biomérieux, Marcy l'Etoile, France) containing 5% sheep blood.³³ Haemolysis was defined as a distinct zone of clearing around and under isolated bacterial colonies after three or 18 hours at 37°C.

MEASUREMENT OF CELL INTEGRITY AND MULTINUCLEATING EFFECT

Cell integrity was determined by measuring release of lactate dehydrogenase (LDH) from cells in the culture medium (Enzyline LDH kit; Biomérieux, Dardilly, France). Infection was conducted with the bacterial suspension of *E. coli* (1×10^8 cfu/well) or with the filtered spent culture supernatants (0.22 μ m filter unit, Millex GS; Millipore, Saint Quentin, France) GmbH). Aliquots (20 μ l) of the incubation medium were sampled one, two, three, or four hours after infection. Results are given as percentage LDH released calculated as follows: LDH release (UI) found in test well/LDH release (UI) found in control well after cell lysis with distilled water (3000 UI). Assays were run at least in triplicate for three successive cell passages. Multinucleating effect due to CNF was studied on HeLa cells and on Caco-2/TC7 cells, as described by de Rycke and colleagues.³⁴ Briefly, a 100 μ l volume of a cell suspension containing 4×10^4 cells/ml (HeLa cells) or 6×10^5 /ml cells (Caco-2/TC7 cells) was distributed in culture cell well plates (Corning Glass Works, Corning, New York, USA) with a glass coverslip, and 22.5 μ l of a fourfold dilution of the bacterial extracts in PBS was added. The multinucleating effect was observed after 72 hours of incubation after the

cells were stained (Giemsa stain) by light microscopy (Leica Aristoplan, Rueil-Malmaison, France).

CELL CULTURE

The cultured Caco-2/TC7 clone cells³⁵ established from the parental human colonic adenocarcinoma Caco-2 cell line^{36, 37} were used. Cells were routinely grown in Dulbecco's modified Eagle's minimal essential medium (25 mM glucose) (Eurobio, Paris, France), supplemented with 10% inactivated fetal calf serum (Boehringer, Mannheim, Germany) and 1% non-essential amino acids. For inhibition of cell association and cell invasion of *S typhimurium*, monolayers of Caco-2/TC7 cells were prepared in six well Corning tissue culture plates (Corning Glass Works). Cells were seeded at a concentration of 1.4×10^4 cells/cm². Maintenance of cells and all experiments were carried out at 37°C in a 10% CO₂/90% air mixture. The culture medium was changed daily. Cells were used at late post confluence—that is, after 15 days in culture. HeLa cells were cultured at 37°C with 5% CO₂ in RPMI 1640 with 2 mM L-glutamine (Life Technologies, Cergy-Pontoise, France) supplemented with 10% inactivated fetal calf serum.

INFECTION OF CACO-2/TC7 CELLS

The cell infection assay was conducted as previously reported.³⁸ Briefly, prior to infection, the Caco-2/TC7 monolayers were washed twice in PBS. Monolayers were then incubated for 15–30 minutes with PBS before infection. A suspension of 2×10^8 cfu/ml (0.5 ml) of bacteria in PBS and 0.5 ml of the cell culture medium (containing 1% mannose to prevent pili-1 mediated adhesion) were added to each well of the tissue culture plate. The plates were incubated for different times at 37°C in 10% CO₂/90% air and washed three times with sterile PBS. *S typhimurium* internalisation was determined by quantitative determination of bacteria located within the infected monolayers using the aminoglycoside antibiotic assay. After one hour of infection, monolayers were washed twice with sterile PBS and incubated for 60 minutes in medium containing 100 µg/ml of gentamicin to kill extracellular bacteria. The monolayers were washed three times with PBS and lysed with sterilised distilled water. Appropriate dilutions were plated on SS to determine the number of viable intracellular bacteria by bacterial colony counts. Assays were conducted several times with three successive passages of Caco-2/TC7 cells.

INHIBITION ASSAY OF *S TYPHIMURIUM* ENTRY WITHIN CACO-2/TC7 CELLS

Inhibition of *S typhimurium* C5 cell invasion by *E coli* was determined as follows: *S typhimurium* (1×10^8 cfu/ml) suspended in PBS with the *E coli* strain in its supernatant (1×10^8 cfu/ml) were added to each Caco-2/TC7 culture well for one hour at 37°C. For the EM0 strain, the experiment was conducted in the presence of dextran 4 (Sigma) at a final concentration of 30 mM for osmotic protection against the pores induced by *E coli* EM0 haemolysin,³⁹ for one

hour at 37°C. Control experiments were conducted with PBS, pH 7. Determination of viable intracellular *S typhimurium* was conducted as described above.

DETECTION OF ANTIBACTERIAL ACTIVITY

Detection of colicin was performed as previously described.¹¹ Briefly, aliquots of 12 hour cultures of *E coli* EM0 or JM105 were placed on hydrophobic membranes (0.45 µm) (HGMF QA Life Science Inc., San Diego, USA) which were themselves placed on brain heart infusion agar plates (Difco). After incubation for 24 hours at 37°C, membranes were removed and plates were overlaid with 10 ml of agar. This overlay agar consisted of minimum medium containing 6 g/l of agar, inoculated with a fresh overnight culture of *S typhimurium* C5. After incubation for 18–24 hours at 37°C, the clear zone surrounding the membranes indicated the presence of antibacterial compounds. Co-culture of *S typhimurium* C5 and *E coli* strains EM0 or JM105 were performed by incubating approximately 1×10^8 cfu/ml of each bacteria at 37°C in LB broth. Initially, and at predetermined intervals, aliquots were removed, serially diluted, and plated on SS agar to determine bacterial colony counts, as described above.

MICE

Both germfree (Cesal, Orléans, France) and conventional mice (Iffa Credo, L'Arbresle, France) were adult female C3H/He/OuJco mice, 7–8 weeks of age. They were housed, fed, and sacrificed in accordance with the highest standards of humane animal care and the relevant national legislation. Germfree mice were reared in Trexler type isolators fitted with a rapid transfer system (La Calhène, Vélizy Villacoublay Cedex, France). Germfree mice were checked for freedom from bacterial contamination by culture of fresh faeces aerobically and anaerobically. They were given ad libitum a commercial diet RO3 40 (UAR, Villemoisson/Orge, France) sterilised by gamma irradiation (40 kGy) and autoclaved demineralised water. Conventional mice were fed an identical but not sterile diet.

INFECTION OF MICE

Germfree mice were infected orally by drinking a *Salmonella* suspension in bottled water. Animals were deprived of water from the day before. Each mouse drank approximately 5 ml of the suspension containing 4×10^5 cfu/ml (that is, approximately 2×10^6 cfu/mouse) within a few hours. This protocol was used to avoid the risks of injuring with an intragastric gavage needle (difficult to manage in isolators) and oral and systemic inoculation. *E coli* EM0 or JM105 strains were inoculated in germfree mice in a similar manner as that for *Salmonella*. Where the effect of colonisation of *E coli* on establishment of C5 was studied, *Salmonella* was inoculated seven days after *E coli* inoculation. Germfree mice infected by one strain only, *Salmonella* or *E coli*, were termed monoassociated mice. Diassociated mice harboured two bacterial strains (*E*

coli and *Salmonella*). Mono or diassociated germfree mice were termed gnotobiotic mice.

Conventional mice were infected intragastrically via a gavage needle. On day 0, mice received either 0.2 ml of LB broth (control) or 0.2 ml of *E coli* culture (assay, 10^8 cfu/mouse). A few minutes later they were infected with a single oral dose of *S typhimurium* C5 (1×10^8 cfu/mouse). For seven days after infection of C5, treatment with LB broth or *E coli* culture was administrated daily at the same dose as on day 0.

DETERMINATION OF *S TYPHIMURIUM* OR *E COLI* IN FAECES AND TISSUES OF MICE

Fresh faecal samples were collected from the anus of each mice. Faecal samples were obtained one, four, and seven days after infection in C5 infected conventional mice. They were collected daily in the case of gnotobiotic mice. Faeces were weighed and diluted 10-fold in PBS. Viable C5 bacteria were determined by plating serial decimal dilutions on SS agar medium (Difco) to differentiate *Salmonella* counts from those of the resident enterobacteria, as described above. Results are expressed as the mean value of viable *Salmonella* counts (log cfu/g of faeces).

Mono or diassociated mice were killed by cervical dislocation. Tissue samples were obtained in the following order: mesenteric lymph nodes (MLN), spleen, and liver. The intestines were unfolded gently to collect MNL and a sterile swab was passed over the intestinal cavity to verify that the intestinal wall had not been damaged. This swab was soaked in 1 ml of PBS which was plated on SS medium. Whenever it proved positive (>5 cfu/ml), the results of the corresponding mouse for MNL, spleen, and liver were discarded. The different segments were removed in the following order: small intestine (divided into three segments corresponding approximately to the duodenum, jejunum, and ileum) and caecum. In most experiments, only the contents were sampled. However, in one experiment the intestinal wall of the segment was obtained. After removal of the contents, the intestinal wall was gently washed with eight successive 5 ml sterile PBS aliquots and drained before being weighed. All content samples were weighed and diluted 10-fold in PBS. Organs were weighed and homogenised with 2 ml of PBS by Ultraturrax for two minutes and diluted 10-fold. The number of viable bacteria in the samples was estimated by plating serial decimal dilutions on TSA or SS agar when differential counts of *S typhimurium* and *E coli* were necessary. Bacterial counts are given per gram of intestinal wall per organ (MLN, spleen, and liver) or per gram of contents.

STATISTICAL ANALYSIS

Numbers of viable bacteria were compared by variance analysis using the Student's *t* test. Numbers of surviving mice were compared by the exact Fisher test.⁴⁰

Figure 1 Polymerase chain reaction (PCR) amplification of *hly*, *cnf*, and *cdt* from *E coli* EM0 strain. Lane 1: 100 bp molecular weight marker; lanes 2–4: *hly* primers; lanes 5–7: *cnf* primers; lanes 8–10: *cdt* primers; lane 11: 1 kb molecular weight markers; lanes 3, 6, and 9: *E coli* EM0; lanes 4, 7, and 10: *E coli* JM105; lane 2: *E coli* SE124, *hly*⁺; lane 5: *E coli* J96, *cnf*⁺; lane 8: *E coli* DH5apOMEO1, *cdt*⁺. The *E coli* strain EM0 showed specific amplification with the *hly* and *cnf* primers whereas the *E coli* JM105 strain showed no amplification.

Results

CHARACTERISTICS OF THE *E COLI* STRAINS

Although the *E coli* strain EM0 was isolated from the faeces of a healthy human volunteer and was described as a non-pathogenic strain,¹³ we found that it had virulence factor genes such as haemolysin (*hly*) and cytotoxic necrotising factor (*cnf*). As shown by PCR (fig 1), the *E coli* strain EM0 showed specific amplification with the primers *hly* and *cnf* as did the positive control *E coli* strains J96, *hly*⁺ and *cnf*⁺, and SE124, *hly*⁺. In contrast, no amplification was found with the *cdt* primers. In addition, *hly*, *cnf*, and *cdt* were not detected by PCR in *E coli* JM105 strain.

Functional haemolysin was revealed by observation of blood haemolysis on sheep blood Columbia agar plates. Concurrently, we found that cell lysis developed in EM0 infected Caco-2/TC7 cells. Indeed, cell infection was followed by release of intracellular LDH in the culture medium which develops as a function of time after infection. In EM0 infected cells at two and three hours after infection, 23 (2)% and 98.7 (6)% of intracellular LDH was released, respectively. The two positive control *E coli* strains J96 and SE124 promoted similar LDH release. Observation of infected cells showed that the monolayers become fragile at one hour after infection and were entirely destroyed at three hours after infection. The culture supernatants of the EM0 strain and the haemolysin positive control strains were inactive, indicating bacterial contact dependent cell lysis.

Cytotoxicity related to CNF has been reported to be characterised by the appearance of multinucleated cells.³⁴ In agreement with this, we observed that infection by the *E coli* strains EM0 and J96 both in HeLa and Caco-2/TC7 cells was followed by the appearance of a high level of multinucleated cells (not shown).

ESTABLISHMENT OF *E COLI* STRAINS IN THE INTESTINE OF MONOASSOCIATED C3H/HE/OUJCO MICE

To study intestinal colonisation in germfree C3H/He/Oujco mice by *E coli* EM0 and JM105

Table 1 Translocation and distribution in the gut of E coli JM105 or EM0 in monoassociated mice

	E coli (mean (SEM)) log ₁₀ cfu/g of:			
	Intestinal content		Washed intestinal wall	
	EM0	JM105	EM0	JM105
Si1 ^a	5.0 (0.6)	5.5 (0.9)	3.8 (0.4)	3.8 (0.6)
Si2	5.9 (0.4)	6.8 (0.4)	3.8 (0.6)	5.6 (0.6)
Si3	7.2 (0.6)	8.3 (0.3)	5.4 (0.5)	7.4 (0.3)
Caecum	10 (0.2)	10 (0.0)	7.6 (0.3)	8.4 (0.2)
Log ₁₀ cfu/organ				
MLN	2.2 (0.1)**	1.3 (0.1)**		
Spleen	0	0		
Liver	0	0		

^aThe small intestine was divided into three equivalent parts (Si1, Si2, Si3) corresponding approximately to the duodenum, jejunum and ileum.

MLN, mesenteric lymph nodes.

**Significantly different, $p < 0.01$.

Figure 2 Survival of control germfree mice ($n=12$) and ex germfree mice monoassociated with E coli JM105 ($n=12$) or EM0 ($n=12$), after S typhimurium C5 oral infection. C5 oral infection (2×10^6 cfu/mice) occurred eight days after inoculation of E coli. Significant difference between control germfree group and EM0 and JM105 diassociated groups ($p < 0.0001$).

strains, two groups of six mice, reared in separate isolators, received E coli EM0 or JM105 bacteria as a single dose (10^6 cfu/ml). Each strain became rapidly established in the gut. EM0 and JM105 bacteria were detected at a level of 9.7 (0.0) and 9.4 (0.0), respectively, (mean (SEM) log₁₀ cfu/g of fresh faeces) one day after oral inoculation, and at 10.5 (0.1) and 10.3 (0.1) on and after day 2 post inoculation. By day 8 after inoculation, mice were killed and the population levels of E coli EM0 and JM105 were determined in intestinal contents (table 1). The two E coli populations similarly increased from the proximal to the distal intestine. E coli EM0 and JM105 were also detected in MLN at about 100 and 50 bacteria per

Table 2 Comparative kinetics of colonisation of S typhimurium C5 in germfree control mice and in monoassociated mice with E coli EM0 or JM105

	<i>Salmonella</i> C5 (mean (SEM)) log10 cfu/g of fresh faeces Days after C5 oral infection			
<i>Germfree mice</i>	1	2	3	5
Control group (n=6) ^a	9.1 (0.2)	9.5 (0.2)	9.8 (0.1)	—
Associated with:				
EM0 (n=12) [*]	6.9 (0.1)	7.6 (0.1)***	8.1 (0.1)***	9.5 (0.1)
JM105 (n=12)**	7.6 (0.1)	9.1 (0.2)	9.6 (0.1)	9.8 (0.2)

^aAs the animals were dying, no more faecal samples were obtained at five days.

^{*}EM0 group significantly different from control group ($p < 0.001$) on days 1, 2, and 3 after infection; ^{**}JM105 group significantly different from control group ($p < 0.001$) on day 1 after infection;

^{***}significant difference between JM105 and EM0 groups ($p < 0.001$).

organ, respectively, indicating translocation. The number of tissue associated E coli was measured in washed organs. E coli EM0 and JM105 were only partly associated with the mucosa of the gastrointestinal tract of monoassociated mice as the number of associated bacteria was always 10 or 100 times lower. This association on the mucosa increased from the proximal to the distal intestine, as for intestinal contents. The number of E coli in aliquots of the last wash of each organ was always 10^2 – 10^4 times lower than the number of E coli remaining in the washed organ itself. Although the EM0 strain has two specific virulence genes, such as haemolysin and CNF demonstrated by PCR, and shows functional effects in vitro, no adverse effect was observed in germfree mice colonised by this strain in our experimental conditions, as previously reported by others.^{9 12 22}

EFFECT OF ESTABLISHMENT OF E COLI STRAINS ON THE MORTALITY INDUCED BY S TYPHIMURIUM AND ON INTESTINAL COLONISATION OF S TYPHIMURIUM C5 GERMFREE C3H/HE/OUJCO MICE

We examined the effect of each E coli strain established in the gut of germfree C3H/He/Oujco mice on S typhimurium C5 induced mortality. Two groups of 12 germfree C3H/He/Oujco mice, reared in separate isolators, were inoculated with EM0 or JM105 E coli. Eight days after E coli inoculation, they were orally infected with S typhimurium strain C5 (2×10^6 cfu/mouse) and compared with a group of 12 germfree mice infected singly by C5 under the same conditions. As shown in fig 2, establishment of E coli EM0 or JM105 in germfree mice significantly increased survival of C5 infected mice. Indeed, at 13 days after infection, all diassociated C5 infected mice survived while all control C5 infected mice were dead ($p < 0.0001$). The EM0 strain apparently gave better protection than the JM105 strain as 11 of 12 and nine of 12 mice, respectively, survived 58 days post C5 infection. However, the difference between the EM0 and JM105 groups was not significant.

EFFECT OF ESTABLISHMENT OF E COLI STRAINS ON INTESTINAL COLONISATION OF S TYPHIMURIUM C5 IN GERMFREE C3H/HE/OUJCO MICE.

The kinetics of establishment of S typhimurium in faeces of EM0 or JM105 monoassociated mice (12 per group) were determined and compared with those obtained in the control germfree group (six mice) after infection by C5 (table 2). On the first day after infection, the level of C5 in faeces was significantly lower in both EM0 and JM105 E coli diassociated groups compared with the C5 infected germfree control group ($p < 0.0001$ and $p < 0.001$, respectively). Afterwards, the level of C5 in faeces evolved differently for each group of E coli monoassociated mice. This inhibition was maintained in the EM0 associated group because by day 5 after C5 infection, the C5 population level had increased and reached the level observed on day 2 after infection in the germfree infected control group. In contrast, for JM105 associated mice, inhibition was no

Table 3 Intestinal colonisation and translocation of *S typhimurium* C5 in germfree mice associated or not with *E coli* EM0 or JM105, three days after C5 infection, and in surviving mice at 58 days after infection

Germfree mice 3 days after infection	Salmonella C5 (mean (SEM))				Log10 cfu/organ		
	Log10 cfu/g of content				Log10 cfu/organ		
	Si1 [§]	Si2 ^a	Si3 ^b	Caecum ^d	Mln ^c	Spleen ^d	Liver ^c
Control group (6)	3.3 (0.5)	5.1 (0.2)	6.6 (0.2)	9.8 (0.1)	5.4 (0.3)	3.4 (0.2)	3.8 (0.1)
Associated with EM0 (5)	3.6 (0.4)	3.6 (0.3)	5.1 (0.4)	8.3 (0.1)	4.2 (0.1)	1.4 (0.3)	2 (0.2)
JM105 (4)	3.2 (0.8)	4.7 (0.6)	4.9 (0.4)	8.6 (0.1)	4.4 (0.1)	1.8 (0.3)	2.5 (0.2)
At 58 days after infection (surviving mice)							
EM0 (5)	nd	nd	5.9 (0.5)*	8.8 (0.2)**	2.4 (0.5)	2.2 (0.3)	3 (0.5)
JM105 (7)	nd	nd	7.3 (0.3)	10.1 (0)	2.5 (0.4)	2.6 (0.4)	3.5 (0.7)

§See legend to table 1.

^aSignificant difference between germfree and EM0 group ($p < 0.01$); significant difference between germfree and both EM0 and JM105 *E coli* groups (^b $p < 0.01$, ^c $p < 0.001$, ^d $p < 0.0001$, respectively); significantly different from JM105 group (* $p < 0.05$ and ** $p < 0.001$, respectively).

nd, not done.

longer significant by days 2 and 3 after infection.

The translocation rate and levels of C5 in the contents of the successive digestive segments were determined in two groups of *E coli* associated mice and in control germfree mice on day 3 after infection by C5 (table 3). Increasing levels of C5 were found from the Si1 segment to the caecum of the control group and from those of the two groups of *E coli* diassociated mice. However, a significant difference was observed between C5 levels in segments Si2, Si3, and the caecum of EM0 diassociated mice compared with C5 infected control mice. In JM105 diassociated mice, the population level of C5 decreased significantly only in distal digestive segments (Si3 and the caecum). The translocation rate of C5 was lowered significantly in both EM0 and K-12 JM105 diassociated mice compared with the control C5 infected group. No significant difference was observed between the two groups of *E coli* associated mice.

In surviving JM105 or EM0 monoassociated C5 infected mice at day 58 after infection, the Si3 and caecal C5 population, and tissue translocation of C5 were determined (table 3). The intestinal and caecal population level of C5 was significantly lower in the EM0 associated mice group than in JM105 associated mice. In contrast, the translocation rate of C5 was identical for both groups of *E coli* associated mice. When examining the level of *E coli* in the faeces of gnotobiotic C5 infected mice from day 5 to day 58 after C5 infection, we found that the populations of *E coli* remained at a similarly high level, which varied from 9.2 (0.1) on day 5 to 8.8 (0.2) log 10 cfu/g of faeces on day 58 for the EM0 monoassociated C5 infected group. In the case of the JM105 monoassociated C5 infected group, populations of *E coli* decreased from 9.1 (0.2) on day 5 to 8.2 (0.4) log 10 cfu/g of faeces on day 58.

EFFECT OF *E COLI* STRAINS ON THE INTESTINAL POPULATION LEVEL OF *S TYPHIMURIUM* C5 IN INFECTED CONVENTIONAL C3H/HE/OJCO MICE
To determine if the decrease in C5 excretion observed in JM105 and EM0 monoassociated C5 infected germfree mice developed in the

presence of a resident microflora, we used three groups of six conventional C3H/He/Oujco mice. Concomitantly with infection with *S typhimurium* C5 (1×10^8 cfu/mouse), conventional mice were orally inoculated daily with one of the *E coli* strains (1×10^8 cfu/day/mouse) or LB broth for the control group. Faeces were collected one, four, and seven days after infection. Figure 3 shows that the level of viable C5 bacteria in faeces was not significantly different in faeces of *E coli* treated C5 infected groups compared with the untreated control C5 infected group. This result demonstrated that in contrast with the germfree situation, *E coli* strains JM105 and EM0 did not decrease *Salmonella* excretion in conventional mice.

E COLI STRAINS DO NOT EXPRESS ANTIBACTERIAL ACTIVITY AGAINST *S TYPHIMURIUM* IN VITRO

Enterobacteriaceae strains exert inhibitory activities to closely related bacteria by producing either microcins or colicins.¹⁰ We have previously reported that *Lactobacillus casei* GG and *L johnsonii* La1 strains produced an antibacterial substance that was active in vitro against

Figure 3 Faecal excretion of *S typhimurium* C5 from conventional mice treated daily with *E coli* strains EM0 and JM105, after C5 oral infection. Control and treated mice were infected with a single dose of *S typhimurium* C5 (1×10^8 cfu/mouse) on day 0. The treatment consisted of 0.2 ml of LB broth (control) or *E coli* culture (2×10^8 cfu/mouse) and was given on day 0 and then daily for seven days after infection. Infection and treatment were achieved using a gastric probe.

Salmonella.^{20 41 42} These two *Lactobacillus* strains, once established in the gastrointestinal tract of germfree mice, can protect them against *S typhimurium* infection and accelerate faecal elimination of *Salmonella* from the gut of conventional mice treated with these bacterial cultures.^{20 42} We investigated if the *E coli* strains EM0 and JM105 could produce antimicrobial substances by two methods. Firstly, co-culture of *E coli* EM0 with *S typhimurium* C5 in LB showed no inhibition of *Salmonella* growth. The inoculum of 7×10^7 cfu/ml of each strain grew simultaneously and reached the level of 4×10^8 and 6×10^8 (log 10 number of cfu/ml) for C5 and EM0, respectively, after five hours of culture and 3×10^8 for the two strains after 24 hours of culture. The same growth curve as *Salmonella* was obtained with *E coli* JM105. Growth of C5 co-cultured with *E coli* was not different from growth of C5 alone in LB broth. Moreover, using the method described by Portrait and colleagues,¹¹ it was observed that these two strains did not produce colicin, which is active against *S typhimurium* C5 (V Portrait, personal communication).

We further investigated if *E coli* EM0 or JM105 could prevent invasion of *S typhimurium* C5 within cultured Caco-2/TC7 cells. For EM0 strain, the inhibition assay of C5 cell invasion was conducted in the presence of dextran 4 for osmotic protection against the pore forming lesions induced by the *E coli* EM0 haemolysin.³⁹ Under these experimental conditions, *E coli* EM0 haemolysin induced LDH release of cells was strongly reduced (10% of LDH release versus 100% in control conditions). In the presence of dextran 4, penetration of *S typhimurium* C5 within Caco-2/TC7 cells was identical to that obtained in control conditions: 6.7 (0.1) (mean (SEM) log10 cfu/ml for seven trials). The results show that none of the *E coli* strains (EM0 and JM105) exerted an inhibitory effect against invasion of C5 within Caco-2/TC7 cells. The level of C5 invasion was 6.6 (0.1) and 6.6 (0.2), respectively, in the presence of JM105 and EM0.

Discussion

Our results showed that virulent and non-virulent *E coli* strains established in the intestine of germfree mice exerted a protective effect against *Salmonella* infection. In contrast, no protective effect was found in the conventional mouse model, suggesting that the resident microflora by itself exerts a protective activity and does not allow the display of the protective activity exerted by *E coli*. These results are different from those recently reported by us showing that the *L casei* GG and *L johnsonii* La1 strains established in the gut of germfree mice delayed the mortality of mice orally infected with *S typhimurium*. In the conventional mouse model, elimination of *Salmonella* from the gut was accelerated in groups treated daily with the GG or La1 cultures^{20 42} compared with the control group. These *Lactobacillus* strains produced an antibacterial substance(s) which was active in vitro against *Salmonella*.⁴¹⁻⁴³ However, the mechanism by which these *Lactobacillus* exerted a protective

effect in vivo against bacterial infection has not yet been elucidated. It has been reported that some *E coli* strains secrete antimicrobial substances termed microcins and colicins which are active against Gram negative bacteria¹⁰ such as *S typhimurium*.¹¹ Barrow *et al* showed that some strains of Enterobacteriaceae such as *E coli*, *Citrobacter*, *Klebsiella*, and *Salmonella* can induce growth suppression of their isogenic antibiotic resistant mutants in early stationary phase LB broth cultures.⁸ This taxon specific inhibition was not related to lack of nutrients and was not the result of lysogenic bacteriophage or bacteriocin activity as the strains were isogenic.⁷ It was suggested that the inhibition was supported by a diffusible but labile chemical factor. When examining if the EM0 and JM105 strains produced an antimicrobial substance(s) active in vitro against *S typhimurium*, we found that there was no inhibitory activity of the two *E coli* strains in the co-culture test and diffusion test. As a consequence, we propose that the protective effect developed by these *E coli* in monoassociated animals could be due to mechanisms other than production of an inhibitory substance.

The protective effect induced by the in vivo established *E coli* in germfree mice could be related to occupancy of *Salmonella* attachment sites as attachment is a prerequisite step before invasion. Indeed, we observed that the two *E coli* strains efficiently colonised the gastrointestinal tract of germfree mice. As a consequence, the established *E coli* could form an efficient biofilm of bacteria protecting the intestinal epithelium against attachment of *S typhimurium*, thus reducing the invasion step. However, our results showed that the number of *E coli* associated with the intestinal mucosa was 100 times lower than the number of *E coli* in the luminal contents, indicating that there is no attachment of the bacteria to the intestinal wall itself.^{12 44} *E coli* detected on the washed intestinal wall probably corresponded to the bacteria embedded in the mucus layer, as observed by others.⁴⁵ Moreover, using a cellular model of human enterocyte-like Caco2/TC7 cells which could be infected by *S typhimurium*, we found that the two *E coli* strains had no effect on the invasion capacity of *S typhimurium*. The protective effect of *E coli* strains cannot be explained by competition for the invasion sites of *S typhimurium*.

E coli EM0 was described as a non-virulent strain as it was recovered from a healthy human donor and has been administered to newborns.¹³ In this study, we showed that EM0 had the genes of two virulence factors (haemolysin and CNF) whereas *E coli* K-12 JM105 does not. However, in our experiments, no adverse effect was observed in germfree mice colonised by this strain, as already shown by other authors in germfree piglets and in mice.^{9 13 22} The insertion sites of these toxins could also be species dependent and absent in the intestine of C3H/He/OuJco mice. There is probably no direct interaction in situ between bacteria and enterocytes which would prevent the effect of haemolysin or CNF, since as mentioned above, the two *E coli* strains do not adhere to the

mucosa. Interestingly, it has been shown previously that 6–22% of faecal *E. coli* strains from healthy donors have haemolytic activity^{33–46} without producing pathology in the donors.

Another mechanism usually attributed to probiotics or to bacterial interactions in vivo is competition for nutrient sources. The following results obtained in chickens^{7–47–48} and pigs⁴⁵ show a protective effect against *Salmonella* intestinal colonisation by previous oral inoculation (one day before) of an avirulent mutant strain of *Salmonella*. As they observed it in vitro, the inhibitory effect appears to work across the serovar boundary but is more efficient with antibiotic resistant isogenic strains.^{45–48} The mechanism involved is related to competition for nutrients or electron receptor as resistance obtained is non-immunological and colonisation by the second bacteria is partially or totally reduced⁴⁸ depending on the mutation of the first strain. Our results showed that mice were protected against clinical disease despite the absence of inhibition of colonisation of *Salmonella* except that there was a significant delay in the establishment of C5 during the two first days after C5 oral inoculation. This delay may be the result of adaptation of the *Salmonella* strain to growth conditions in the presence of *E. coli* but competition does not lead to elimination of the strain. Filho-Lima *et al* showed recently that EM0 associated to probiotics (*L. acidophilus* and *Saccharomyces boulardii*) was necessary to intestinal elimination of an antibiotic resistant mutant of *S. flexneri* but had no effect on intestinal levels of *S. enteritidis* subspecies *typhimurium*.²²

Our results showed protection against systemic infection and lower contamination at remote sites such as the liver and spleen without inhibition of intestinal colonisation. These results are similar to those of Barrow *et al* (Prevention of salmonellosis in gnotobiotic pigs by precolonisation with avirulent *Salmonella* strains. “*Salmonella and Salmonellosis*”. *Proceedings of Ploufragan*, 20–22 May 1997:519–20). They showed a protective effect against *S. typhimurium* infection by inoculating gnotobiotic pigs with an avirulent strain of *S. infantis*. Gut colonisation by *S. typhimurium* was delayed for five days in pretreated animals which survived to oral infection. In these surviving pigs, translocation rates of *S. typhimurium* in MLN, liver, and spleen were decreased significantly as we observed in surviving mice pretreated with *E. coli* (tables 3, 4). Diminution of C5 translocation rate observed here in the MLN could be a consequence of diminution of the intestinal colonisation rate in situ, as Berg and Owens described in conventional mice that the level of translocation to the MLN is related to intestinal levels greater than 10⁸/g.⁴⁹ Diminution of *Salmonella* at remote sites (liver and spleen) suggests an immunological mechanism. Rapid intestinal colonisation of *E. coli* one week before infection and delay in *S. typhimurium* establishment could allow progressive development of an immunological mechanism of defence. We showed that both EM0 and

JM105 strains translocated from the gastrointestinal tract to the MLN, as found previously by others for indigenous *E. coli* strains in mice^{50–51} and piglets.^{45–52} The clinical significance of translocation of indigenous bacteria and its role in priming the host immune response to improve host defences against overt or opportunistic pathogen(s) is not yet known.⁵⁰ Some authors have reported that *E. coli* participates in the establishment of the population of IgA plasmocytes in the lamina propria in adult germfree mice, in suckling mice,⁵³ and in germfree piglets⁵² to the level obtained in conventional animals. *E. coli* EM0 has several immunological properties whereas those of *E. coli* K-12 have not yet been well studied. In a germfree mice model,²⁵ EM0 stimulated secretion of the cytokines interleukin (IL)-1 and IL-6 by peritoneal macrophages, to the level observed in conventional mice and cytokine production of bone marrow derived macrophages indicating an effect on macrophage precursors.²⁴ Other strains of *E. coli* had a similar effect in germfree mice exposed to these strains: stimulation of IL-6 and tumour necrosis factor (TNF).⁵⁴ Production of TNF- α was also stimulated by the EM0 strain in gnotobiotic mice.²⁵ This cytokine in conjunction with interferon γ are the main cytokines implicated in the first steps of *Salmonella* infection.⁵⁵ As a consequence, it can be hypothesised that concerning infection of C3H/He/Oujco mice by *S. typhimurium*, the presence of an established *E. coli* in vivo could stimulate a non-specific immunological response.

Further experiments are required to establish the mechanism by which these two virulent and avirulent *E. coli* strains directly affect *S. typhimurium* pathogenicity. In particular, the immunological aspects of the protective role of *E. coli* should be studied.

We thank V Portrait for colicin determination, C Sandré for revision and council in redaction, and M Métioui for his revision of the English language of the manuscript. J Guignot is supported by a doctoral fellowship from the Ministère de l'Éducation Nationale, de la Recherche et de la Technologie (MENRT).

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