BACTERIAL PATHOGENICITY

Survival and distribution of cell-free SEF 21 of *Salmonella enterica* serovar Enteritidis in the stomach and various compartments of the rat gastrointestinal tract *in vivo*

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Rats were dosed for 6 days with purified SEF 21 fimbriae of *Salmonella enterica* serovar Enteritidis 10360. The levels of fimbriae in gut contents associated with tissues and in the faeces were quantified by direct non-competitive ELISA. SEF 21 was distributed throughout the gut. The majority was found in the large intestine where it was primarily in the luminal contents. In contrast, a high proportion of SEF 21 detected in the ileum, the main site of salmonella colonisation and invasion, was tissue-bound. Thus, purified SEF 21 survived intestinal passage and associated with the stomach and gastrointestinal tract in a pattern similar to that found with whole *Salmonella* cells.

Introduction

The incidence of *Salmonella enterica* serovar Enteritidis (*S. Enteritidis*) infection remains high in the UK, the main types being *S. Enteritidis*, PT4 and PT6 [1]. *S. Enteritidis* expresses a plethora of defined fimbriae [2] including SEF 14 [3], SEF 17 [4] and SEF 21 [5]. SEF 14 has been shown to be a T-cell immunogen [6] and to contribute to adherence to mouse epithelial cells [7]. SEF 21 are composed of 21 kDa fimbrin monomers that are virtually identical to the type 1 fimbrin of *S. Typhimurium* but distinct from the type 1 fimbriae of *Escherichia coli*. However, they share the property of mannose-sensitive haemagglutination. The role of SEF 21 remains in question because the factors that affect fimbrial phase variation *in vivo* [8, 9] and influence variability in fimbriation of cells have not been fully elucidated. Therefore, it has been difficult to evaluate the role of SEF 21 in infection.

In salmonellae, type 1 fimbriae are purported to initiate intestinal colonisation and may contribute to tissue

tropism by adhering to specific mannosylated host proteins [10, 11]. Furthermore, type 1 fimbriae of salmonellae are known to mediate binding to a number of epithelial cell lines *in vitro* [12–14]. Recent work has shown that flagella [15] rather than SEF 21 [16] may have a role in the pathogenesis of *S. Enteritidis* in the chicken. This is in agreement with work by Robertson *et al.* [17] who have shown in a rat explant model that flagella may have a role in salmonella infection. It has been demonstrated that mannose-specific SEF 21 and type 1 fimbriae of *S. Typhimurium* associate with the rat small intestine *in vivo* [18], and salmonella infection can be reduced significantly by the inclusion of a mannose-specific lectin in the diet [19]. Overall, this suggests that SEF 21 might be important in the interaction of salmonellae with specific cells or regions of the gut rather than having an all-embracing function as the means of attachment to the gut. SEF 21 was prepared and purified and its ability to survive in the gut of rats and associate with the epithelium *in vivo* was evaluated in this study.

Materials and methods

Bacteria

S. Enteritidis 10306 has been characterised previously [20] and was used to produce purified SEF 21 for experiments *in vivo*.

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Purification of SEF 21

Type 1 fimbriae of *S. Enteritidis* 10360 were purified and characterised as described previously [20]. Briefly, the production strain was grown in 6 L of Heart Infusion Broth (CM225, Unipath, Basingstoke) under static conditions and harvested [21]. The yield of fimbriae after ultracentrifugation and ultrafiltration steps was 4.7 mg/ml of total fimbrial protein. To obtain pure fimbriae, this crude preparation was applied to an HPLC gel filtration column. Fractions were analysed by ELISA and SDS-PAGE, and those containing fimbriae were pooled and stored at -20°C . SDS-PAGE/Western blot profile showed that this preparation contained a 21-kDa doublet of the SEF 21 fimbrial subunit (Fig. 1). Fimbriae purified in this way were used for the immunisation of mice, in selection of monoclonal antibodies (MAbs) after fusion and for *in vivo* studies.

Preparation of antibodies

An IgA MAb to type 1 fimbriae, designated M732, was prepared in mice and fully characterised as described previously [20].

Fig. 1. SDS-PAGE and Western blot of purified SEF 21 extracted from *S. enteritidis*. Lane 1, molecular mass markers (from bottom to top, 14.3, 21.5, 30.0, 46.0, 69.0, 97.4, 200.0 kDa); 2, purified SEF21; 3, SEF 21 probed with anti-SEF 21 chicken sera.

Animal handling and experimental protocol

Eight hooded Lister rats (19-day-old male) from the Rowett colony were divided into two groups (four rats per group) housed singly in metabolism cages and pre-fed for 10 days on a good quality diet based on lactalbumin (protein 100 g/kg diet) as the sole protein source [22]. Feed and water were available *ad libitum*. Once the rats had reached *c.* 85 g in weight, the amount of food offered was reduced to 6.5 g/day per rat (given as two feeds over the day) over a period of 3 days. The rats were then given lactalbumin diet (6.5 g/day per rat) with or without (controls) pure isolated SEF 21 (Fig. 1) added to it for 6 days according to the regimen outlined in Table 1.

The rats were weighed on each day of the experiment. The final dose of type 1 fimbriae (4 mg) in saline (1 ml) or saline alone (controls) was given to the rats by gavage 90 min before they were killed by anaesthetic overdose and exsanguination. A full dissection was performed. Stomach, small intestine, caecum and colon were flushed out with phosphate-buffered saline (PBS, pH 7.2) and the contents were collected and frozen. The small intestine was divided into four pieces – duodenum (0–20 cm), jejunum (20–60 cm), ileum (60–70 cm) and terminal ileum (70–80 cm) – and the sections were snap frozen. Frozen sections and contents were freeze-dried and weighed. Faeces collected (24-h intervals) during the 6-day study were frozen and freeze-dried. All experimental procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986.

Determination of SEF 21 fimbriae in the samples

Samples (tissue, contents and faeces) were extracted in 20 mM diamino-propane (pH 11.4) and homogenised at 19 000 rpm (Ultra Turrax T50) for 5 min on ice. The samples were centrifuged at 10 000 *g* for 25 min and the resultant supernate was taken off. Four extractions were performed on each sample and the resulting supernates were pooled and further centrifuged at 15 000 *g* to obtain a clear supernate. The samples were sub-divided and stored frozen until required. Extractions from the control samples were used as negative controls.

Table 1. SEF 21 regimen

Day	Additions to diet (mg)
1	1.0
2	1.5
3	1.5
4	3.0
5	10.0
6	21.0
Final	4.0
	(by gavage)
Total	42.0

Detection of SEF 21 fimbriae by enzyme-linked immunosorbent assay (ELISA)

Flat-bottomed micro-ELISA plates (Nunc) were filled with 0.1 ml of anti-SEF 21 antibody (M732) diluted 1 in 1000 in carbonate buffer (pH 9.6), and incubated at 4°C overnight. The wells were washed three times with PBS (pH 7.2) and blocked with 0.15 ml of bovine serum albumin (BSA; Sigma) 0.1% in PBS for 30 min at 37°C. After three washes with PBST (PBS containing Tween 20 1% v/v) and two washes with PBS, the sample was added to the wells and serially diluted, then the plates were incubated for 1 h at 37°C. After another washing with PBST to remove unbound sample, 0.1 ml of anti-SEF 21 antibody (M732) conjugated to biotin diluted 1 in 1000 in PBST + BSA was added to the wells and the plates were incubated for 30 min at 37°C. The wells were washed three more times with PBST followed by two washings with PBS to remove free antibodies, before addition of streptavidin peroxidase diluted 1 in 500 in PBST. After incubation for a further 30 min at 37°C, the wells were washed five more times with distilled water before addition of 0.1 ml of the substrate consisting of ready to use 3,3',5,5' tetramethylbenzidine (Univet, Berkshire). After incubation for 5 min at room temp (16°C), the reaction was stopped by addition of 0.05 ml of 1 M H₂SO₄. The yellow colour was measured at 450 nm with a spectrophotometer (Uniskan, Life Sciences, UK). A standard curve was plotted against purified SEF 21 and a regression analysis carried out to determine the SEF 21 content of the samples.

Statistics

ELISA was performed in triplicate on three separate occasions. Values from the four rats in each group were pooled. Data are expressed as the mean and SD.

Results

Analysis of the gut contents and tissues from rats given SEF 21 revealed that SEF 21 fimbriae were found throughout the gastrointestinal tract. The recovery of SEF 21 from the four test rats varied considerably, particularly in relation to the amounts associated with small intestine tissue (Fig. 2). Except for the distal small intestine, most SEF 21 was present in the luminal contents, with only small amounts associated with the tissues. Significant amounts of fimbriae were also recovered from the faeces (Fig. 3).

Stomach

The smallest amount of the SEF 21 was recovered from the stomach. The ratio of tissue-associated: luminal SEF 21 was *c.* 1:9 (Fig. 2).

Small intestine

Higher levels of SEF 21 were present in the small intestine (Fig. 2). Unlike in the stomach, a considerable proportion (*c.* 50%) of the SEF 21 was associated with the tissue, particularly in the ileum, where the recovery

Fig. 2. Cell-free SEF 21 recovered for the stomach and gastrointestinal contents and tissues. Each bar represents the mean and SD; *n* = 4 apart from small intestine (SI) and colon, where *n* = 3. □, Contents; ■, tissue; ▨, jejunal tissue; ▩, ileal tissue.

Fig. 3. SEF 21 recovered from the faeces over 6 days. Each bar represents the mean (and SD) of four rats.

was 1.2 µg/mg dry tissue as compared with 0.62 µg/mg dry weight in the jejunum.

Large intestine and faeces

High amounts of SEF 21 were recovered from the large intestine, particularly the colon. The bulk of the SEF 21 was recovered from the luminal contents (caecum >77%, colon >90%). The level of SEF 21 in the faeces increased over the first 2 days and was relatively stable thereafter (Fig. 3). This was surprising, as daily intake of SEF 21 increased >20-fold between day 2 and day 6. Initially *c.* 30% of the intake of SEF 21 was recovered from the faeces, but by day 6 <4% of intake was detectable in faeces.

Growth of rats

Weight gains by rats given SEF 21 were similar to the control animals (data not shown). Furthermore, the weights of the gut tissues did not differ between the test and control groups.

Discussion

Fimbriae from salmonellae have been purified and characterised before [5, 20, 23] but, to the authors' knowledge, the potential of SEF 21 to compartmentalise and survive passage through the gastrointestinal tract has not been investigated. Indeed, few studies have dealt with the interactions of isolated fimbriae or pili with the gastrointestinal tract. Berendson *et al.* [24]

showed that purified *E. coli* RDEC-1 pili adhered to rabbit intestinal mucosa with the same distribution as whole piliated organisms. Nakasone and Iwanga [25] showed that the ability of purified pili from *Vibrio parahaemolyticus* to adhere to intestine correlated with the role of these pili in colonisation. Furthermore, the isolated pili were adhesive to rabbit intestinal epithelium in the absence of the viable organism [26]. Sherman and Boedecker [27] found that luminal glycoproteins within the mucus layer affected replication and colonisation by *E. coli* RDEC-1 and Mack and Blain-Nelson [28] showed that mucin inhibition of RDEC-1 was region-related and contributed to site localisation of the bacterium and type 1 fimbriae [29]. Furthermore, a purified lectin (PA-1) from *Pseudomonas aeruginosa* has been shown to induce gut growth when ingested by the rat [30].

It has been shown previously that *S. Enteritidis* distributed throughout the rat gastrointestinal tract and that the main site of attachment, colonisation and invasion was the ileum [31, 32] and that SEF 21 was expressed on *S. Enteritidis*, and associated with the terminal ileum *in vivo* [18]. Thus the distribution and tissue association of purified SEF 21 observed in the present study correlated well with the location of *S. Enteritidis* bacteria in infected rats [32].

Isolated SEF 21 survived passage through the stomach to a large part and small amounts were found associated with the tissue. Previous work has shown that viable salmonellae can be recovered from the rat stomach and survive there over a significant period of

time [32]. The recovery of purified fimbriae from the stomach tissue may suggest that less acidic micro-environments occur within the stomach, protecting the fimbriae from stomach acid, allowing association with the stomach tissue. Alternatively, salmonella numbers in the stomach may be a result of limited fermentation events in the stomach [33], which could modulate the stomach environment allowing the survival of salmonellae [34].

The distribution of isolated SEF 21 in the small intestine followed a similar pattern to viable salmonellae expressing SEF 21 in infected rats [32]. In agreement with previous work by the group [18] with immunohistochemical methods, higher levels of tissue-associated SEF 21 were found in the jejunum and ileum than in the stomach or the large intestine. Association of cell-free SEF 21 was exclusive to the distal ileum of the rat. Hence, it is unlikely that peristalsis and flow rates in the gastrointestinal tract had a significant effect on the distribution of SEF 21 in the small intestine. It is possible that high numbers of receptors are available for the association of SEF 21 in the ileum and this would be consistent with high numbers of polymannosylated glycoconjugates in the distal region of the small intestine [35].

Previously, the main site of colonisation and invasion by salmonellae in the rat model was shown to be the distal small intestine [32]. In the present study, the main site of adherence of purified SEF 21 was in that region of the gut. These findings are consistent with a role for SEF 21 in attachment of salmonellae to epithelia of the distal small intestine.

Recovery of isolated fimbriae from the caecum and colon was high. However, the bulk of the isolated fimbriae were in the contents rather than the tissues. In infection studies with rats, the highest numbers of salmonellae were found in the caecum and colon [31, 32]. Furthermore, in pig studies, the salmonellae were found in the lumen and not attached to the gut wall [36]. Hence, *Salmonella* whole bacteria and purified SEF 21 had similar patterns of dispersion throughout the large intestine.

Significant levels of SEF 21 were found in the faeces. This probably reflects the high amounts of fimbriae found in the caecal and colonic contents, suggesting that some of the purified SEF 21 passed through the gut with minimal proteolytic degradation. However, the amounts detected in faeces were low and very variable. Moreover, the mean recoveries between day 2 and day 6 were fairly similar (750–1120 mg/day per rat) despite an approximately 20-fold increase in the daily input of SEF 21 during that period. This suggests that a very high proportion ($\geq 90\%$) of the SEF 21 ingested during the later stages of the study was degraded or taken up by the body. Plant lectins bound to gut epithelial cells have been found to be significantly

more resistant to proteolytic attack than those in luminal contents [37]. Therefore, it is possible that SEF 21 detected in faeces may have been derived primarily from SEF 21 bound to the gut during passage and was thus partially protected from proteolytic attack. Fimbriae in free solution may have been readily degraded. In the absence of major changes to gut structure or function, the amount of SEF 21 able to bind to the gut would be fairly constant, irrespective of input. Furthermore, the amount of SEF 21 coming off the epithelium would depend on the rates of cell shedding, which again in the absence of major functional changes in the gut would be relatively constant.

We have demonstrated that cell-free type 1 fimbriae (SEF 21) from *S. Enteritidis* associate with the intestine in the same manner and in the same regions of the gut as *S. Enteritidis* expressing SEF 21 or type 1 fimbriate *S. Typhimurium* [18]. The capacity of SEF 21 to survive stomach passage and associate with specific regions of the gastrointestinal tract suggests that they may have potential as carriers of heterologous antigen [38]. Furthermore, these findings provide direct evidence that bacterial fimbriae are capable of mucosal adherence in intestine *in vivo* and support the hypothesis that these structures may serve as mucosal adherence factors during the initial stages of enteric infection by *S. Enteritidis*.

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