Quantification of the leucocyte influx into rabbit ileal loops induced by strains of *Salmonella typhimurium* of different virulence

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Summary. Leucocyte influx into rabbit ileal loops, induced by strains of *Salmonella typhimurium* of different virulence, was assessed with ¹¹¹Indium-labelled leucocytes. Strains fell into two groups on the basis of their leucotactic potential: “virulent” strains (which induced fluid secretion) caused a dose-dependent leucocyte influx; strains which did not induce fluid secretion failed to induce a significant leucocyte influx. Fluid secretion was never observed in the absence of leucocyte influx, but leucocyte influx *per se* did not induce fluid secretion. The phenotype of the challenge inoculum influenced fluid secretion; young log-phase organisms induced fluid secretion with a higher frequency than overnight cultures. These findings support earlier evidence implicating leucocytes in an interactive but not exclusive role in the genesis of salmonella-induced fluid secretion. They suggest, though do not prove, that interaction of leucocytes with the appropriate phenotype of organisms results in the release of a host-derived or bacterial secretagogue, or both. The bacterial factor may or may not be the antigen related to cholera toxin, described previously.

Introduction

The mechanisms involved in the induction of fluid secretion by salmonellae are poorly understood, and have been reviewed by Stephen *et al.* (1985). Previously we have shown that “virulence” of *Salmonella typhimurium*—ability to induce fluid secretion in rabbit ileal loops (Wallis *et al.*, 1986a; Clarke *et al.*, 1988)—does not correlate with ability to make enterotoxin *in vitro* (Wallis *et al.*, 1986a) or ability simply to adhere to intestinal tissue (Worton *et al.*, 1989).

Subjective histological evidence for the possible involvement of leucocytes in the genesis of fluid secretion was obtained by Giannella *et al.* (1973), who examined various strains of *S. typhimurium* in the rabbit ileal loop test (RILT)—a good model for *S. typhimurium* diarrhoeal disease (Stephen *et al.*, 1985). Giannella and co-workers found that strains TML, W118 and M206 invaded rabbit ileal mucosa

and induced fluid secretion; such strains were designated biotype (+ +) by Clarke *et al.* (1988). Strains SL1027 and LT7 invaded the ileal mucosa but induced negligible fluid secretion—biotype (+ –); strain Thax-1 neither invaded the mucosa nor induced fluid secretion—biotype (– –). Examination of histological sections suggested that strains TML and W118 induced an intense, but unquantified, inflammatory cell influx into infected intestinal mucosa, whereas strain Thax-1 did not evoke such influx; strains M206, SL1027, and LT7 evoked an intermediate inflammatory reaction (Giannella *et al.*, 1973). We have re-examined these strains in the RILT and, with the exception of strain M206, which in our hands is of biotype (+ –), they have retained their biological properties (Wallis *et al.*, 1986a). Giannella (1979) demonstrated the negative corollary to the data on inflammatory cell influx: in rabbits rendered neutropaenic with nitrogen mustard, salmonella-induced fluid secretion in RILs was inhibited.

Additional indirect evidence, possibly implicating polymorphonuclear leucocytes in the genesis of fluid secretion, stems from the use of indomethacin which inhibits the synthesis of prostaglandins. The latter are generated by several cell types, including

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neutrophils, during the inflammatory process (Higgs *et al.*, 1975) and have been demonstrated to induce fluid secretion in the intestinal mucosa (Pierce *et al.*, 1971; Matuchansky and Bernier, 1973). Two types of experiment have been performed previously.

First, the fluid response to challenge with live organisms was studied in RILs and monkeys treated with indomethacin. Gots *et al.* (1974) showed that if rabbits were pre-treated with indomethacin, fluid secretion in ileal loops challenged with *S. typhimurium* was almost completely inhibited, whereas bacterial invasion and the accompanying inflammatory cell influx appeared normal. Fluid secretions evoked by *Vibrio cholerae*, *Shigella flexneri* and cholera toxin (CT) were also reduced by 50%. Giannella *et al.* (1977) also showed that salmonella-induced diarrhoea in orally inoculated monkeys could be inhibited by prior treatment with indomethacin.

Second, Giannella *et al.* (1975) examined also the levels of adenylate cyclase in RILs challenged with CT and with *S. typhimurium* strains TML (+ +) and SL1027 (+ -). CT and strain TML caused increase in adenylate cyclase activity and also fluid secretion, whereas strain SL1027 did not. Indomethacin pre-treatment inhibited the increase in adenylate cyclase activity and fluid response in RILs challenged with strain TML. In the case of CT, adenylate cyclase activity was not reduced but the fluid response was reduced by 50%. Giannella *et al.* (1975) proposed that the previously reported failure of strain SL1027 (+ -) to induce more than a mild inflammatory cell influx (Giannella *et al.*, 1973) was the reason for its failure to induce fluid secretion. These data question the exclusivity of the role played by adenylate cyclase, and hence cyclic nucleotides, in both CT- and *S. typhimurium*-induced fluid secretion. They suggest a role for an indomethacin-sensitive (possibly prostaglandin) mediator, though one cannot assume that the indomethacin acted only upon prostaglandin synthesis.

In contrast to the above, Duebbert and Peterson (1985) denied a role for inflammatory cells in the induction of fluid secretion, and refocussed attention on the possible role of a CT-like salmonella-enterotoxin acting *via* cyclic adenosine monophosphate (cAMP) and prostaglandins originating from epithelial cells. However, first, their data (in their table 1) give no evidence that cAMP or prostaglandins are involved in the induction of fluid in RILs challenged with *S. typhimurium*; there were no significant differences in the levels of either cAMP or prostaglandins produced by fluid-inducing and

non-fluid-inducing strains. Moreover, the degrees to which these levels were depressed by indomethacin were similar for both fluid-inducing and non-fluid-inducing strains. Second, we have shown that (+ -) and (+ +) strains produce enterotoxin(s) *in vitro* equally well; and we have argued that enterotoxigenicity, at least as determined *in vitro*, cannot *per se* be the sole factor which determines fluid secretion by *S. typhimurium in vivo* (Wallis *et al.*, 1986a).

In this laboratory, we have attempted to resolve this controversy. In a previous study, with the same strains as those used by Giannella's and Peterson's groups, we observed consistently that the onset of fluid secretion in RILs coincided with an inflammatory cell influx into the ileal mucosa (Wallis *et al.*, 1986b). In the present paper we describe experiments to quantify the influx of leucocytes into RILs challenged with strains of different virulence, in an attempt to strengthen or weaken the possible correlation between fluid secretion and a role for leucocytes. The approach is based on techniques used by clinicians to locate internal abscesses by use of radio-labelled leucocytes (Coleman *et al.*, 1980).

Materials and methods

Bacterial strains

Six strains of *S. typhimurium* (TML, W118, LT7, SL1027, M206 and Thax-1) have been used previously (Wallis *et al.*, 1986a; Clarke *et al.*, 1988), and their biotypes are stated in the *Introduction*. Strain WAKE was isolated from a patient in an outbreak of *S. typhimurium* food poisoning at Stanley Royd Hospital, Wakefield, and was kindly donated by Leeds Regional Public Health Laboratory; it was of biotype (+ +) in the RILT. Strains were stored at -70°C in equal proportions of glycerol and Muller-Hinton broth.

Rabbits

New Zealand White rabbits (2.5-3 Kg) were obtained from Regal Rabbits, Great Bookham, Surrey, and from Ranch Rabbits, Crawley Down, Sussex. All were bled on arrival, and their sera screened for antibodies to *S. typhimurium* by slide agglutination, and to CT by immunoblotting (Clarke *et al.*, 1988).

Preparation of inocula

Inoculum A, as used initially, was prepared as follows. Strains were streaked on MacConkey's agar and incubated at 37°C overnight. A small portion of each of c. 15 colonies was suspended in 10 ml of sterile saline, and

10 ml of Hartley Digest Broth (HDB; Oxoid) were inoculated with 25 μ l of this suspension. After incubation at 37°C overnight, organisms were washed in sterile isotonic saline (NaCl 9 g/L in distilled water), and resuspended in fresh HDB; then $c. 1 \times 10^9$ viable bacteria were inoculated into RILs which were constructed and challenged as described previously (Wallis *et al.*, 1986a).

Inoculum B. The above protocol was modified midway through this work—see Qi *et al.* (1989) and *Results and Discussion*. HDB was inoculated heavily from $c.$ five colonies on a MacConkey-agar plate, and incubated for only 4.5 h. Again, $c. 1 \times 10^9$ viable washed bacteria, suspended in fresh HDB, were used to challenge the RILs.

Positive control loops were inoculated with 1 μ g of CT in 1 ml of sterile saline.

Negative control loops were inoculated with 1.5 ml of sterile HDB. Results were considered valid only if negative control loops were flat and CT loops had V/L (volume (ml)/length (cm) of loop) ratios >0.5 ; usually the V/L ratios of CT loops were in the range 1.5–2.5 ml/cm.

Immunofluorescence microscopy

Full thickness mucosal biopsies were sampled from ileal loops and were fixed, sectioned and stained by an

indirect fluorescent-antibody method to detect salmonellae as previously described (Wallis *et al.*, 1986b).

Quantification and imaging of the inflammatory cell influx

The white-cell labelling method was a modification of that used by Hawker *et al.* (1985)—see fig. 1. The experimental protocol was designed such that the labelled leucocytes were re-injected just before the expected leucocyte influx into the intestinal mucosa, which occurred reproducibly in this experimental model (Wallis *et al.*, 1986b).

Four hours after challenge of the loops, 17 ml of blood were taken from an ear vein of each animal and added to 3 ml of acid citrate ($\text{Na}_3\text{citrate}$ 25 g/L, citric acid, anhydrous, 13.6 g/L; pH 4.9) in a polystyrene universal container (Sterilin 128/B); 5 ml of dextran (mol. wt $c. 500\,000$) 6% w/v in isotonic saline were then added to the blood, which was gently mixed and allowed to stand at 37°C for up to 1 h to permit erythrocyte sedimentation. Sedimentation was allowed to occur sufficiently to yield $c. 10$ ml of erythrocyte-free supernate which contained plasma, leucocytes and platelets; and 8 ml of this supernate was transferred to a round-bottomed, polystyrene tube (Sterilin, 142AS) with a plastic pasteur

Fig. 1. Schematic diagram for the leucocyte labelling protocol.

pipette (Appelton Woods, Birmingham). White cells were sedimented by centrifugation (640 *g*, 5 min at room temperature); the supernate, containing plasma and platelets, was collected and held at room temperature for use as described below. The pellet, comprising mixed leucocytes and platelets, was suspended in 10 ml of Ca^{2+} -free Tyrode's buffer (NaCl 8 g/L, KCl 2 g/L, NaHCO_3 1.0 g/L, $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ 0.05 g/L, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.4 g/L, D-glucose 1.0 g/L; pH 6.5) containing prostaglandin E_1 (Sigma) 0.4 $\mu\text{g}/\text{ml}$, the latter being included to inhibit platelet aggregation. The leucocytes were recentrifuged (640 *g*, 5 min, room temperature) and resuspended in 2 ml of Ca^{2+} -free Tyrode's buffer containing prostaglandin E_1 .

^{111}In indium oxine (Amersham International, Bucks) 100 μCi was added to the leucocyte suspension and incubated for 2 min at room temperature; 8 ml of platelet-rich plasma were then added to the leucocyte suspension to absorb any unbound isotope; and the leucocytes were recentrifuged (640 *g*, 5 min, room temperature). Finally, leucocytes were resuspended in 5.5 ml of Ca^{2+} -free Tyrode's buffer (without prostaglandin E_1) and reinjected into the test rabbit through a 21G hypodermic needle *via* an ear vein; experiments showed that the final suspension contained less than 15 ng of prostaglandin E_1 . The procedure from bleeding to reinjection took c. 2 h.

Animals were sedated by intravenous injection of pentobarbitone 30 mg/kg (Willington Medical, Shrewsbury), 12 h after initial challenge with organisms, and a gamma-scan of the animals was performed with a Gamma-camera—Searle LFOV Camera fitted with a medium energy collimator and information stored on an on-line DEC (PDP) computer. The rabbits were then killed by intravenous pentobarbitone overdose, and the V/L ratios were determined (Wallis *et al.*, 1986a). Ileal loops were excised and trimmed of mesentery and sutures. Mucosa and loop-contents were then counted separately in a NaI well crystal attached to a rate meter (Nuclear Enterprises, Reading, Berks) set to detect the two photopeaks of ^{111}In at 173 and 247 keV. The leucocyte influx into the test loops was measured as a ratio of cpm for test loop to cpm for negative control loop in the same rabbit (leucocyte influx ratio).

Results

Screening of rabbit sera

Of the rabbits used in this study, 50% had agglutinating antibodies to *S. typhimurium*. In contrast to those used by Clarke *et al.* (1988), none of our rabbits had circulating antibodies to CT. There was no correlation between fluid secretion in response to (++) strains, or to CT, and the presence of antibodies to *S. typhimurium*.

Rabbit imaging

Fig. 2 shows a typical gamma-camera ventral image of a rabbit with ^{111}In -labelled leucocytes and

Fig. 2. A typical gamma-camera ventral image of a rabbit with ^{111}In -labelled leucocytes, 12 h after ileal loop challenge. Two test loops (*) challenged with *S. typhimurium* strain TML can be seen in the lower abdominal region. The heart (H), liver (L) and spleen (S) can also be seen, as well as a fainter skeletal image (shoulder and knee).

S. typhimurium-infected ileal loops. Light areas representing regions of high ^{111}In activity can be seen in the liver, spleen and lower abdomen. The areas of high activity in the lower abdomen represent the two ligated ileal loops infected with strain TML.

Bacterial invasion as assessed by immunofluorescence microscopy

Subjective examination of mucosa challenged with (++) strains, and sampled at 12 h, revealed many luminal as well as intracellular salmonellae. Though villi were not all equally invaded, the majority were heavily invaded with up to 50 fluorescently stained bacterial foci seen in sections; a few villi showed few or no invading bacteria.

Although fluid secretion did not occur in all loops that were inoculated with (++) strains, there was no obvious difference in the degrees of invasion by

these strains, between loops which had fluid secretion and those which did not.

Quantification of leucocyte influx induced by CT and by different biotypes of S. typhimurium

In fig. 3, the data have been compiled from 126 loops constructed in 57 rabbits inoculated with either type A (overnight) or type B (log phase) inocula and observed after 12 h. Strains TML, W118 and WAKE were more leucotactic than strains LT7, SL1027, M206 and Thax-1. Only 48% of the rabbits responded by secreting fluid (V/L = 0.5–3.2 ml/cm) into RILs challenged with

strains TML, W118 and WAKE, of biotype (+ +)—see below; but the (+ -) strains gave consistently negative results (V/L < 0.5 ml/cm). In loops challenged with CT, in which an intense fluid response was observed (V/L = 1.5–2.7 ml/cm), the mean leucocyte influx ratio was 2.4.

Because, in the initial experiments with inoculum A, the varied fluid response did not correlate with the serological status of the animals, two changes were made in an attempt to increase the proportion of animals giving the expected fluid response to (+ +) strains. First, animals were left for 24 h before examination; but this did not increase the proportion of loops with a positive fluid response, though

Fig. 3. Leucocyte influxes into RILs induced by strains of *S. typhimurium* of different virulence and by cholera toxin, 12 h after loop challenge: ● = loop challenged with inoculum A (overnight growth); ○ = loop challenged with inoculum B (early log-phase). The mean leucocyte influx and standard error of the mean are indicated for strains TML, W118, WAKE and LT7, and cholera toxin. The data for strains SL1027, M206 and Thax-1 were tightly grouped and therefore the means are not indicated.

$$\text{Leucocyte influx ratio} = \frac{\text{cpm for loop challenged with bacteria (or toxin)}}{\text{cpm for negative control loop}}$$

it did increase the leucocyte influx into loops challenged with (+ +) strains and CT (fig. 4). Second, animals were challenged with young log-phase organisms (inoculum B) and examined at 12 h: there was a marked increase in the number of loops showing fluid secretion. This is most clearly shown by re-analysis of the data in fig. 3, as presented in figs. 5a and 5b showing the leucocyte influxes in loops in which fluid did or did not accumulate. For strains TML and W118 combined, 73% of all the responders were challenged with inoculum B; only 10% of the non-responders were challenged with inoculum B. The mean leucocyte

Fig. 4. Leucocyte influxes into RILs (with and without fluid accumulation), 24 h after loop challenge. Loops were challenged with inoculum A (overnight growth) of *S. typhimurium* strains TML and W118 and with cholera toxin. The mean leucocyte influx (see fig. 3) and SEM are indicated for each strain.

Fig. 5. Leucocyte influxes (see fig. 3) into ileal loops 12 h after loop challenge. Re-analysis of data in fig. 3. Loops were challenged with *S. typhimurium* strains TML and W118: ● = loop challenged with inoculum A (overnight); ○ = loop challenged with inoculum B (log-phase); a, loops without fluid accumulation; b, loops with fluid accumulation. The mean leucocyte influx and SEM are indicated for each strain.

influx with secreting loops was marginally greater than for non-secreting loops.

The relationship between size of inoculum B and leucocyte influx is shown in fig. 6. There was variation between individual rabbits (in rabbit 4, the leucocyte influx was less intense compared with rabbits 1, 2 and 3), but for each rabbit there was a correlation between leucocyte influx and inoculum size. In addition, with one exception, fluid secretion was observed only in loops challenged with inocula $\geq 10^7$ organisms.

Discussion

We report studies on the host response in experimental *S. typhimurium* infection of ligated RILs, with strains characterised previously and a recent clinical isolate, and a novel application of a technique used routinely in clinical medicine. To our knowledge, this is the first time that virulence of *S. typhimurium*—at least as demonstrated by fluid secretion in RILs—has been correlated with a quantifiable parameter. We found two groups of strains: TML, W118 and WAKE were potent inducers of a leucocyte influx; LT7, SL1027, M206 and Thax-1 did not induce a leucocyte influx significantly above background. These findings thus support the possibility that white cells play a role in the causation of fluid secretion, because only strains TML, W118 and WAKE caused significant fluid secretion in the RIL.

It is clear, however, that a leucocyte influx *per se* does not induce fluid secretion, since in a large number of experiments (figs. 4 and 5a) high influxes of leucocytes occurred with no associated fluid secretion. This failure to secrete could not be correlated with the presence of anti-salmonella antibodies in the sera of the animals.

By comparison with the (++) strains, CT induced only a slight influx of leucocytes at 12 h, while inducing an intense fluid response. An increased leucocyte influx was seen at 24 h, probably in response to mucosal damage resulting from elevated hydrostatic pressure developing within such ileal loops over the longer period.

Three points emerge which might throw some light on the mechanism(s) of fluid secretion. First, despite the fact that leucocyte influx *per se* is not directly responsible for salmonella-induced fluid secretion, the latter was never observed in the absence of a significant leucocyte influx. Second, the phenotype of the fluid-inducing strains is obviously important—young cultures induced secretion with a strikingly higher frequency than older cultures (figs. 5a and b). Third, the size of the initial inoculum is important because, with one exception, loop dilation occurred only when the inoculum was $\geq 10^7$ cfu/challenge (fig. 6).

We therefore postulate that leucocyte influx into infected intestinal mucosa plays an important part in the genesis of fluid secretion. Interaction of leucocytes with bacteria of the correct phenotype results in the release of a host-derived secretagogue (possibly prostaglandins of neutrophil or other cell origin) or of a bacterial enterotoxin, or both. The data do not allow discrimination between these two possible mechanisms, involving or not involving

Fig. 6. Leucocyte influxes (see fig. 3) into rabbit ileal loops in four rabbits (R1–R4): dose response induced by inoculum B (log-phase) of (++) biotype of *S. typhimurium* (strains TML and W118). Volume/length (V/L) ratios: – = <0.3; ± = 0.3–0.4; + = 0.5–0.9; ++ = 1.0–1.5; +++ = >1.5 ml/cm.

release of prostaglandins; nor need they be mutually exclusive. Whichever is the case, there is clearly an optimal bacterial phenotype since young log-phase cultures are more efficient elicitors of fluid secretion than organisms taken in late-log or stationary phase. We have not yet proved the existence of a CT-like enterotoxin, as demonstrated by Finkelstein *et al.* (1983); but already, in the strains used in this study, we have shown the existence of a salmonella enterotoxin (Wallis *et al.*, 1986a), and a CT-related antigen (Clarke *et al.*, 1988) which may or may not be a CT-related toxin. The expression of this antigen is dependent on the culture medium and the time at which the culture is examined (Clarke *et al.*, 1988; Qi *et al.*, 1989). There is a higher proportion of organisms bearing this antigen in early log-phase than at subsequent stages of growth. In retrospect, we now see that the biotyping described by Wallis *et al.* (1986a), in which there was a high degree of reproducibility, was done with inocula of early log-phase organisms. The change to overnight cultures was a logistical convenience, which at first did not appear to affect adversely the

ability of (+ +) strains to elicit fluid secretion. Clearly the initial success with older cultures was fortuitous, because subsequently this was not routinely repeatable. The CT-related antigen is at least a useful marker of the fully virulent (i.e., fluid-inducing) phenotype of (+ +) strains. It may yet prove to be a determinant of fluid secretion.

Thus, it could be that influx of neutrophils is induced by invading organisms, and that fluid secretion arises from the interaction of these leucocytes with luminal organisms to release a toxin or an endogenous secretagogue, or both. The

bacterial phenotype is apparently more important for the genesis of fluid secretion than invasion of the intestinal mucosa: subjective examination of immunofluorescently stained sections of infected mucosa showed that old and young cultures invaded to the same degree, irrespective of fluid secretion; but these observations on invasion need to be quantified. Tests of these hypotheses have begun.

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