The role of leucocytes in the induction of fluid secretion by *Salmonella typhimurium*

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Summary. Nitrogen mustard (N₂M) treatment of rabbits induced neutropenia, and, in ligated ileal loops, it inhibited fluid secretion induced by salmonella or by cholera toxin (CT). Pretreatment of rabbits with indomethacin almost abolished salmonella-induced fluid secretion and significantly reduced that induced by CT. Similar effects of N₂M and indomethacin on fluid secretion induced by salmonella, but not by CT, have been reported by other workers and used to implicate prostaglandins, from the salmonella-induced inflammation, as mediators of fluid secretion. In contrast, we show that N₂M treatment, in addition to reducing CT-induced secretion, caused severe morphological alterations to ileal mucosa. Irradiation techniques were developed for inducing neutropenia, but they did not totally inhibit salmonella-induced leucocyte influx into ileal mucosa. We propose an alternative mechanism for the inhibitory effect of N₂M on salmonella- and CT-induced secretion, based on the known anti-mitotic activity of N₂M. Also, the anti-secretory effect of indomethacin cannot be attributed uniquely to its anti-inflammatory activity because it depressed CT-induced secretion as well as salmonella-induced secretion. These results support the concept of pathophysiological secretion in infectious diarrhoea, developed previously for rotavirus and extended to bacterial infections.

Introduction

In a study by Wallis *et al.* (1989) a correlation was shown between virulence of *Salmonella typhimurium*—as expressed by fluid secretion in ligated rabbit ileal loops (RILs; Clarke *et al.*, 1988)—and ability to evoke an influx of leucocytes into RILs challenged with the organism. This strengthens the claim, disputed by some, that leucocytes (with a high neutrophil content in this experimental system) are involved in the causation of diarrhoeal disease induced by *S. typhimurium*; for a summary of the arguments, see Wallis *et al.* (1989).

However, proof that neutrophils play a vital role in the induction of such fluid secretion would require experiments in rabbits rendered neutro-

penic by acceptable means. Alternatively, one could predict that interference with appropriate neutrophil function in an otherwise normal animal would inhibit salmonella-induced fluid secretion if this were neutrophil-mediated.

In this study, we have repeated experiments originally described by Gianella (1979), using nitrogen mustard (N₂M) to render animals neutropenic. Also, as an alternative to N₂M, we have tried bone-seeking radio isotopes with or without X-irradiation. In addition, we have used indomethacin (a known inhibitor of prostaglandin synthesis), though we were aware of the interpretative problems associated with the use of pharmacological inhibitors (Peterson *et al.*, 1988; Wallis *et al.*, 1989).

Materials and methods

Bacteria

Two strains of *S. typhimurium* (TML and W118) were used. Both are inducers of fluid secretion in ligated RILs (Wallis *et al.*, 1986a; Clarke *et al.*, 1988).

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Animals

New Zealand White rabbits (2.5–3 kg) were obtained from Regal Rabbits, Great Bookham, Surrey, and Ranch Rabbits, Crawley Down, Sussex.

Leucocyte counts

From an ear vein of the test rabbit, 2.5 ml of blood were dripped into an EDTA-blood tube (Pink, 2.5-ml potassium EDTA tubes from Midland Laboratory Equipment, Lichfield). Blood films were made on microscope slides, fixed in methanol 100% for 2 min and stained as follows: May Grunwald's stain (BDH; 0.25% w/v in absolute methanol), 2 min; Giemsa stain (BDH; 10% v/v in Giemsa buffer), 5 min; Giemsa buffer (pH 6.8; buffer tablet, BDH, in 1 L of water), 5 min. The film was examined by light microscopy, and differential counts of 50 leucocytes (monocytes, neutrophils and lymphocytes) were recorded.

For total leucocyte counts, 20 μ l of blood sample were added to 90 μ l of a solution of triton X-100 (Sigma) 0.2% in phosphate-buffered saline (NaCl 8.5 g/L, Na_2HPO_4 1.07 g/L, NaH_2PO_4 0.39 g/L; pH 7.1) containing ethidium bromide (Sigma) 50 mg/L, and the mixture was left for 15 min at room temperature. The leucocyte concentration was determined in a haemocytometer by ultra violet microscopy with 546-nm exciter filters. From total and differential leucocyte counts, concentrations of circulating neutrophils, monocytes and lymphocytes in test animals could be monitored.

Induction of neutropenia with N_2M

This was based on methods described by Giannella (1979). Test rabbits were anaesthetised with pentobarbitone, and injected with N_2M 1.75 mg/kg intravenously. Total and differential leucocyte counts were performed before and 72 h after N_2M treatment.

Also at 72 h after N_2M treatment, ileal loops were constructed and inoculated with approximately 5×10^8 cfu of *S. typhimurium* strain TML or W118 (Wallis *et al.*, 1986a). Sterile culture medium (Hartley Digest Broth, HDB) and cholera toxin (CT) 1.0 μ g were used as negative and positive controls respectively. After 18 h the V(ml)/L(cm) ratio was measured for each loop and biopsy specimens were taken for light microscopy, fluorescent antibody (FA) microscopy, and transmission and scanning electron microscopy (TEM and SEM)—see Wallis *et al.* (1986b).

Induction of neutropenia with radio-isotopes and X-irradiation

Based on previous work with mice (Emmanuel *et al.*, 1981), bone-seeking isotopes were used in the hope of destroying haemopoietic cells in the marrow, thereby inducing leucopenia. The complex procedures for generating and administering the isotopes, for handling and

holding of live animals, and for disposing of radioactive cadavers, were carried out according to the provisions of the Radiation Safety Unit in the University of Birmingham.

Ideally, isotopes should be incorporated rapidly and specifically into bone tissue, should emit intense β -radiation of sufficient energy (> 1 MeV) to irradiate the local bone environment, and should be short-lived so that activity declined rapidly after injection and allowed safe handling of animals.

Three radio-isotopes (Vaughan *et al.*, 1987) were examined. Theoretical levels of activity necessary to induce neutropenia with each isotope were calculated with a computer model, based on parameters such as isotope half-life, rate of isotope incorporation in bone, and rate of isotope excretion.

$^{18}\text{Fluoride}$ (^{18}F) has a half-life of 1.8 h, emits both positrons and annihilation γ -rays and is bone-seeking. Rectilinear imaging of animals revealed that 80% of the injected isotope was incorporated in the skeleton within 90 min of injection. However, neutropenia was not induced.

$^{152\text{M}}\text{Europium}$ ($^{152\text{M}}\text{Eu}$) was also tried because it is a bone-seeking element (O'Mara *et al.*, 1969), but it was abandoned because it did not induce neutropenia. Analysis of femur and rib bones (see below) revealed incorporation of only 16% of injected radio-isotope into the skeleton, and this was the probable reason for failure to induce neutropenia.

$^{177}\text{Lutecium}$ (^{177}Lu), with a half-life of 6 days, emits both β -particles and γ -rays; it was demonstrated by O'Mara *et al.* (1969) to be bone-seeking, and we confirmed this with rectilinear imaging. To a vial containing ^{177}Lu (produced by thermal neutron bombardment of natural lutecium in the Risley Research Reactor, Manchester) 1.0 ml of 1M HCl was added, and the temperature of the vial was raised to 80°C for 2 h to dissolve the isotope. After cooling, 0.154 g of nitrotriacetic acid chelator was added, and the solution was diluted with 9.6 ml of phosphate buffered saline pH 7.2. Rabbits were sedated by pentobarbitone, and each received 40 mCi of ^{177}Lu intravenously. Before injection and at 48-h intervals thereafter, animals were bled from an ear vein, for total and differential leucocyte counts. At the end of the experiment, animals were killed, samples of rib and long bone (c. 1 g) were taken, and the activity in each bone sample was measured with a γ -counter. From these data, the level of isotope incorporation into the whole skeleton was calculated to be 60% of the total injected, corresponding to a total marrow dose of approximately 1650 centigray (cGy) in one week (1 cGy $\equiv$ 1 Rad).

Combination of ^{177}Lu and X-irradiation. Injection of ^{177}Lu induced only a transient drop in leucocyte count, despite the incorporation of 60% of the injected isotope into the skeleton. This probably resulted from inability of the ^{177}Lu β -particles to traverse the full width of the rabbit long bones. The lack of trabeculae in these areas allows marrow distant from the bone surface to escape irradiation. To overcome this, the long bones were subjected to external X-irradiation.

Animals were sedated with pentobarbitone and their abdomens shielded with lead (2.5-cm thickness) resting on supports on either side of the animal. The animal's head, thorax, fore-limbs, hind-limbs and pelvis were irradiated with 3-mm copper 1/2 value layer X-rays, irradiating at 265 kV and 12 mA at a distance of 0.5 m from the shutter. Animals were first laid on one side and irradiated for 20 min, then the irradiation was stopped and the animals were turned over and irradiated for a further 20 min. The radiation dose to the abdominal region was measured with a Farmer Baldwin dosimeter; the monitoring probe was placed inside the lead shielding, near the mid-abdominal region, for the duration of the irradiation procedure. The protected abdomens received a radiation dose of only 5 cGy. Next day, animals were given injections of ^{177}Lu , as already described. Five animals were used in this experiment; three received both X-ray and ^{177}Lu treatment, and two control animals were given natural Lu. The total circulating leucocyte counts in test rabbits and controls were monitored.

Use of indomethacin to modify fluid secretion

The method used was that described by Gots *et al.* (1974) with minor modification. Indomethacin 0.5 g was dissolved in 10 ml of ethanol 100% by sonication (Hilsonic water bath, Chromatography Services Ltd, Wirral) for 30 min, and added to 90 ml of 0.05M KH_2PO_4 , pH 8.0. Starved rabbits were given this indomethacin solution (10 mg/kg) by intravenous injection, 3 h before inoculation of loops and twice thereafter at 7-h intervals to give a total dose of 30 mg/kg. Five 10-cm ileal loops were constructed; two were inoculated with *c.* 5×10^8 cfu of *S. typhimurium* (strain TML or W118), two with sterile HDB and one with 1.0 μg of CT. The same culture of strain TML and the same batch of CT were used to provide identical inocula for ileal loops constructed in rabbits treated with the same ethanol- KH_2PO_4 solution which contained no indomethacin. The animals were killed 21 h after loop challenge; the V/L ratios of the loops were determined, and full-thickness mucosal biopsy specimens were taken and fixed for examination by light- and FA-microscopy, and by SEM and TEM.

Other methods

Fluorescent-antibody staining of salmonella, SEM, TEM, histology of ileal mucosa by light microscopy, preparation of inocula, ligation of loops and measurement of V/L ratios were all performed as described by Wallis *et al.* (1986a,b; 1989).

Results

Effects of N_2M on rabbits

Table I shows the reduction in circulating leucocyte counts in N_2M -treated animals. Fig. 1 shows the response of six N_2M -treated and four normal animals to challenge with *S. typhimurium* strains TML and W118. N_2M treatment completely inhibited fluid secretion induced by strain W118, and reduced by *c.* 80% that induced by strain TML ($p < 0.05$). CT-induced fluid secretion was reduced by *c.* 45% ($p < 0.01$).

Tissue sections from infected loops in normal rabbits showed many invading bacteria within enterocytes involving the majority of villi (fig. 2a); loops in N_2M -treated rabbits showed a dramatic increase in bacterial invasion (fig. 2b).

After staining with haematoxylin and eosin (HE), sections from negative control loops in normal rabbits showed typical ileal histological appearances (fig. 3). Corresponding sections from N_2M -treated animals exhibited many abnormal features (fig. 4a, b). There was, as expected, an absence of neutrophils in blood vessels and mucosa; the section shows also reduction in villus height, pronounced irregularity in section profile, and shedding of villus cells and degenerate cell contents into the lumen. Changes of cell organisation were also evident in N_2M -treated villi: though subjectively the numbers of lymphocytes seemed to be the same as in normal controls (not shown), the distribution of lympho-

Table I. Influence of N_2M -treatment of rabbits on numbers of circulating leucocytes

Blood cells	Mean cell count* (SEM) in		
	four untreated control rabbits	eight N_2M -treated rabbits	
		before treatment	after treatment
Total leucocytes	10.0 (0.7)	11.9 (1.0)	2.0 (0.2)
Neutrophils	3.5 (1.1)	2.6 (0.5)	0.1 (0.05)

* $\times 10^6$ cells/ml of blood.

Fig. 1. Fluid responses in rabbit ileal loops challenged with strains TML and W118 and with cholera toxin in normal and N₂M-treated rabbits. Each closed circle or open square represents one loop. The means and SEMs are indicated.

cytes appeared to be random throughout the tissue (fig. 4a and inset). The highly abnormal architecture of N₂M-treated villi was also seen by SEM (fig. 5a, b). N₂M-treated villi were generally flattened vertically and shrunk laterally, and they had narrow tips and were more flaccid.

In loops constructed in normal animals, challenged with strain TML or W118, the following was observed: the expected (Wallis *et al.*, 1986b) massive influx of leucocytes (predominantly neutrophils), and shortened villi with little or no extruded villus debris overlying the mucosa (fig. 6); these loops invariably contained fluid. Corresponding loops in N₂M-treated animals showed a significant reduction in the number of infiltrating leucocytes, gross mucosal damage similar to or usually greater than that shown in fig. 4, and huge areas of degenerate material in close proximity to residual mucosa which had entrapped within it large numbers of bacteria (fig. 7); these loops contained little, usually no, fluid.

Use of ¹⁷⁷Lu with X-irradiation to modify fluid secretion

Although N₂M did reduce leucocyte influx and did inhibit fluid secretion, it also damaged the gut

Fig. 2. Fluorescent-antibody stained sections of ileal mucosa challenged with strain TML in (a) control and (b) N₂M-treated rabbits. In (b) the brightness of fluorescence renders the background relatively indistinct; the lumen (L), sub-mucosa (SM), crypt (C) and brush border mucosal surface (white arrowheads) are marked. Mucosa from N₂M-treated rabbits was much more heavily invaded with salmonellae (white areas) than mucosa from control rabbits challenged with same dose of bacteria from the same batch culture. Bars = 100 μ m.

Fig. 3. HE stained section of ileal mucosa from loop challenged with sterile HDB in normal rabbit. Villi looked normal and healthy with no gross abnormalities. Bar = 100 μ m.

in a manner which flawed the experiment. An alternative means of rendering animals neutropenic was therefore sought.

One of the three Lu + X-ray-treated rabbits died 2 days after treatment, from an unknown cause. In the other two, the total circulating leucocyte counts were reduced *c.* 10-fold, compared with the two control animals, 6 days after the treatment (fig. 8) and too few leucocytes were present on the blood films for an accurate differential count.

Five days after treatment, animals which received ^{177}Lu + X-ray treatment and normal animals were starved for 24 h, and ileal loops were then challenged with strain TML. The fluid response was determined 18 h later, and it differed in the two "leucopenic" rabbits (table II). In one rabbit, the response was not significantly different from that in the normal rabbits; in the other rabbit, it was almost completely inhibited. However, the luminal contents of the salmonella-challenged loops in

Table II. Fluid response in ligated ileal loops challenged with strain TML of *S. typhimurium* or with cholera toxin in rabbits treated with ^{177}Lu and X-irradiation

Inoculum	Fluid secretion (V/L)* in ileal loops of			
	control rabbit 1	control rabbit 2	test rabbit 1	test rabbit 2
5.5×10^8 cfu of strain TML	{ 3.8 2.7	{ 1.0 1.1	{ 3.0 2.3	{ 0.1 0.2
1.5 ml of sterile saline	0	0	0	0
1.0 μ g of cholera toxin	1.6	3.7	2.9	2.8

*Volume of fluid (ml)/length of loop (cm).

"leucopenic" rabbits were purulent, indicating that a leucocyte influx into the infected mucosa had occurred. FA-stained sections of infected mucosa showed that bacterial invasion was similar in the treated and normal animals. HE-stained sections confirmed that a leucocyte influx into the infected mucosa of treated rabbits had occurred, although it was less than in the normal rabbits.

This experiment was repeated; but the animals were left for longer, to allow a greater drop in circulating leucocytes. This did not occur: leucocyte counts were depressed to the same degree for 14 days, after which they began to rise. Further experiments, with increased doses of ^{177}Lu and X-rays, failed to produce complete inhibition of leucocyte infiltration.

Use of indomethacin to modify fluid secretion

A summary of the fluid responses is shown in fig. 9. *S. typhimurium* strains were tested in seven normal rabbits and nine rabbits treated with indomethacin, with two test loops in each animal. Indomethacin reduced salmonella-induced fluid secretion by 75–90% ($p < 0.05$). CT was tested in three control and two indomethacin-treated rabbits, with one loop in each rabbit; indomethacin reduced CT-induced secretion by *c.* 45% ($p < 0.02$).

The degrees of bacterial invasion and leucocyte influx in normal and indomethacin-treated rabbits were again compared by subjective examination of two sections from each loop, both stained by FA and HE. No obvious difference in the degrees of bacterial invasion was observed (results not shown). The degrees of leucocyte influx were similar but the gross appearance of the mucosa was different. In indomethacin-treated rabbits, leucocytes present in

Fig. 4. HE stained section of ileal mucosa from loop challenged with sterile HDB in N_2M -treated rabbit; (a) and (b) represent one full-thickness section of rabbit ileum from serosa (S) to lumen (Lu), with four separate overlapping fields combined into two composites; (a) overlaps with (b) along the horizontal black line. Note the absence of neutrophils in blood vessels and mucosa. The gross architecture of villi was highly abnormal: vilus height was reduced and section profile irregular; considerable amounts of degenerate tissue (D) overlay the mucosa. Cellular organisation within residual villi was also abnormal. Lymphocytic cells (small, dark round cells; L, inset) appeared to be distributed randomly and to be present in very large numbers. Normal untreated tissue at similar magnification (not shown) revealed similar cells in similar quantities but located mainly in the lamina propria or in interepithelial cellular spaces. Despite the extensive damage, N_2M -treated loops were not secreting fluid. Bar = 40 μ m.

the ileal lumen were more closely associated with the mucosal surface than those in normal rabbits. This was probably a result of salmonella-induced fluid secretion in the untreated rabbits washing the luminal debris, including leucocytes, from the luminal surface. No shortening in the length of villi in infected loops of treated animals was observed.

Discussion

In agreement with the findings of Giannella (1979), we have shown that rabbits may be rendered leucopenic by N_2M -treatment, and that the ability of such animals to mount a secretory response to challenge by virulent strains of *S. typhimurium* is almost completely abolished. However, in two important respects, our data differ from those of Giannella. First, as judged both by light microscopy and SEM, the morphology of uninfected villi treated with N_2M was very different from that of untreated

villi. However, despite the considerable degree of architectural damage, and high numbers of invading bacteria of strains that induce fluid secretion in normal animals (Wallis *et al.*, 1986b), no significant fluid secretion occurred. Second, fluid secretion induced by CT was reduced significantly, by 45%. Hence, caution is needed in interpreting the lack of neutrophil influx, induced by N_2M , into loops challenged with *S. typhimurium* as direct evidence for the involvement of neutrophils in a normal fluid response.

The basic premise in the use of N_2M is the assumption, claimed by Giannella (1979) to have been fulfilled, that N_2M does not damage or upset the gut in a manner prejudicial to the experimental design; clearly in these experiments it did. However, N_2M is used therapeutically in the treatment of certain neoplasias by virtue of the alkylating effect on DNA and hence on dividing cells (Salmon, 1980). It is, therefore, possible that the reduction of

Fig. 5. SEM appearance of rabbit ileal mucosa taken from negative control loop in N_2M -treated and normal rabbits. Compared with normal control villi (**b**), N_2M -treated villi (**a**) were greatly reduced in height, narrowed at the tips, shrunk vertically and laterally, and flaccid. Bar = 100 μm .

villus height and other morphological changes are a direct consequence of N_2M affecting crypt cell mitoses. This would affect normal epithelial cell replacement and hence height of villi, and also crypt functions which by general consensus (Roggin *et al.*, 1972; Field *et al.*, 1980; Welsh *et al.*, 1982) are involved in normal secretion. Increased rates of villus base/crypt cell division are involved in the hypersecretory phase of rotavirus diarrhoea (Collins *et al.*, 1988; Spencer *et al.*, in press) and have been implicated in other bacterial toxin-induced diarrhoeas (Stephen and Osborne, 1988). Clearly N_2M was not acting as a general cytotoxic agent, since it did not affect the numbers of long-lived lymphocytes.

Giannella (1979) reported no change in CT-induced fluid secretion in N_2M -treated rabbits; here we do. As outlined above, this unexpected result for CT provides more evidence that interference with crypt cell division (see Kimberg *et al.*, 1973) results in significant but not complete reduction of CT-induced fluid secretion in acute

Fig. 6. HE stained section of ileal mucosa from loop challenged with strain TML in a normal rabbit. Note the abundance of neutrophils in blood vessel, submucosa, mucosa, and lumen. No haemorrhage was observed and villi were markedly shortened. These observations are highly typical of all such loops in these and previous experiments (Wallis *et al.*, 1986b). Bar = 40 μm .

experiments and supports the views of Stephen and Pietrowski (1986) on the mode of action of CT.

Clearly, N_2M is not ideal for rendering animals neutropenic in these experiments. Much effort was made with bone-seeking isotopes but only partial neutropenia was achieved. Use of ^{177}Lu in conjunction with X-irradiation to inhibit haemopoiesis resulted in a 10-fold reduction of circulating leucocytes. However, in the few challenge experiments, the fluid response in one animal was accompanied by an infiltration of neutrophils (test rabbit 1; table II): the luminal contents were visibly purulent and the mucosa infiltrated with leucocytes, albeit less than in the controls, as judged by histological examination. This contrasts with N_2M -treatment in which a similar (6-fold) reduction in total leucocytes occurred with a 26-fold reduction in circulating neutrophils, and a near total inhibi-

Fig. 7. HE stained section of ileal mucosa from loop challenged with strain TML in a N_2M -treated rabbit. The overall appearance of challenged N_2M -treated loops was similar to or, usually, worse than that shown in fig. 4. This higher magnification shows also clumps of bacteria entrapped in tissue debris in close association with the mucosa; such loops contained little fluid, usually none. Bar = 6 μ m.

Fig. 8. Effect of ^{177}Lu + X-ray combined treatment on number of circulating leucocytes. Each count is the mean of readings from two rabbits, expressed as cells/ml of blood: total leucocytes in control rabbits (●) and in ^{177}Lu -treated rabbits (○); neutrophils in control rabbits (■) and in ^{177}Lu -treated rabbits (□); * = cells in the blood film too few to count.

tion of neutrophil infiltration into the challenged mucosa.

The indomethacin data substantiate the findings of Gots *et al.* (1974) with *S.typhimurium* and CT. However, the present data and those of Wallis *et al.* (1989) suggest that if prostaglandins are involved in either *S.typhimurium*- or CT-induced fluid secretion, they are unlikely to be of neutrophil origin.

Fig. 9. Fluid responses in rabbit ileal loops challenged with strains TML and W118 and with cholera toxin in seven normal and nine indomethacin-treated rabbits, expressed as means and SEMs. Loops challenged with overnight growth (●) or log-phase culture (○) of *S.typhimurium* or with cholera toxin (□)—for details of inocula, see Wallis *et al.* (1989).

This view rests on the following assumption and reasoning. If *S.typhimurium* and CT share a common or partially common mechanism for inducing fluid secretion, then that common part does not involve neutrophils: there is no evidence for a

necessary influx of neutrophils in CT-induced secretion. Also, in every case examined here and by Wallis *et al.* (1989), either quantitatively or qualitatively, virulent strains elicited a leucocyte influx but not always a fluid response. It seems unlikely that neutrophil-bacterial interaction, which has been postulated by Giannella (1979) as giving rise to the release of prostaglandin secretagogues, occurs only when secretion is induced: neutrophil-bacterial interactions probably occur in every case of control and indomethacin-treated rabbits challenged with *S.typhimurium*. Therefore, an indomethacin step common to *S.typhimurium* and CT must be mediated by cells (possibly enterocytes) other than neutrophils, as has been claimed for CT (Duebbert and Peterson, 1985).

We have not yet, by technically satisfactory means, proved the absolute necessity for a neutrophil-influx as part of the biological mechanism in salmonella-induced fluid secretion; the induction of neutropenia is being pursued by immunological

means. However, combination of the present data with those of Clarke *et al.* (1988) and Wallis *et al.* (1989) allows the following synthesis to be made. *S.typhimurium* of the correct virulent genotype invades the intestinal mucosa. This induces an influx of leucocytes, mainly neutrophils. The latter interact with luminal or invading organisms or both, and if of the correct phenotype these release a processed CT-like toxin. This toxin acts *via* an indomethacin-sensitive step, as does CT. Fluid secretion is then elicited. If the indomethacin-sensitive step is one giving rise to prostaglandins with secretagogue activity, the cellular origin of prostaglandin is unlikely to be neutrophils.

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