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Fate of swallowed interleukin 8 and TNFα, and effect on the Wistar rat gut

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Abstract

To evaluate the passage of cytokines through the gastrointestinal tract, we investigated the digestion of interleukin-8 (IL-8) and tumour necrosis factor α (TNFα), in vitro and in vivo, and their propensity to induce intestinal inflammation. We serially immuno-assayed IL-8 and TNFα solutions co-incubated with each of three pancreatin preparations at pH 4.5 and pH 8. We gavaged IL-8, TNFα and marker into 15 Wistar rats, and measured their faecal cytokine concentrations by ELISA and histologically examined their guts. IL-8 immunoreactivity was extinguished by all pancreatin preparations after 1 h of incubation at 37 °C. TNFα concentration progressively fell from 1 to 4 h with all enzyme preparations. Buffer control samples maintained their cytokine concentrations throughout incubation. No IL-8 or TNFα was detected in any rat faecal pellets. There was no significant proinflammatory effect of the gavaged cytokines on rat intestine. IL-8 and TNFα in aqueous solution could well be fully digested in the CF gut when transit time is normal and exogenous enzymes are provided, although cytokines swallowed in viscous sputum may be protected from such digestion. © 2002 European Cystic Fibrosis Society. Published by Elsevier Science B.V. All rights reserved.

Keywords: Interleukin-8; Tumor necrosis factor α (TNFα); Pancreatic enzymes; Wistar rats

1. Introduction

Tentative reports of raised faecal cytokine concentrations have been made following speculativly assayed faecal samples, which used ELISAs validated for use in non-faecal samples [1,2]. Similarly, we [3] and others [4] have reported raised faecally derived cytokine concentrations in patients with cystic fibrosis. In our latter study we suggested that they originated from swallowed sputum, because there seemed to be a relationship between chest disease severity and faecal IL-8 concentration. This stimulated us to speculate that a repeatable non-invasive proxy measure of pulmonary inflammatory severity might be developed. The validity of the ELISA assays for faecally derived IL-8 and TNFα used in these early studies was brought into question, when we found that they paradoxically measure increasing cytokine concentrations with dilution of faecal extract [5]. Thus, the presence of lumenal cytokine immunoreactivity may be a result of intestinal inflammation, swallowed sputum or artefact.

We were therefore interested to ascertain whether pancreatic enzyme preparations commonly used in cystic fibrosis can digest sputum-derived cytokines in conditions likely to be encountered in the CF gut, and whether IL-8 and TNFα can pass through the gut of a pancreatic sufficient mammal in an assayable form, and mediate intestinal inflammation in doses that a CF patient would be expected to swallow.

We chose to gavage the cytokines into the pancreatic sufficient Wistar rat because these rats respond to human recombinant IL-8 [6–9] and TNFα7 [10] with responses that parallel the human response to these cytokines, but the rat IL-8 equivalent chemokine GRO/CINC is immunologically distinct from human IL-8 [11]. This allowed us to be confident that any faecal IL-8 detected in rat faeces originated from the administered human cytokine.

2. Methods

2.1. Enzymatic degradation of interleukin-8 and TNFα

Enzyme solutions of Creon Forte (Solvay), Pancrease (Cilag) and CotazymS (Organon) were prepared to an

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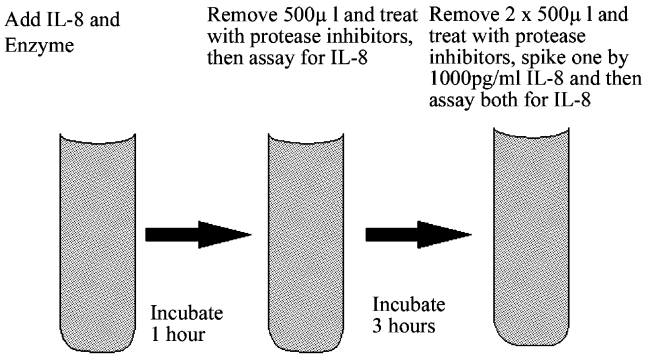

Fig. 1. IL-8 and enzyme coincubation experiment.

estimated concentration of 250 units of lipase activity per ml in both pH 4.5 and pH 8 Tris buffer, and aliquots of these solutions were analysed for tryptic activity.

A 1-ml aliquot of IL-8 (2000 pg/ml) or TNFα (4545 pg/ml) was added to 1 ml of each enzyme solution and control buffer and incubated at 37 °C in a water bath. After 1 h a 500-μl aliquot was removed and the enzyme activity was inhibited by the addition of 50 μl of soy bean trypsin inhibitor (SBTI; 1 mg/ml in phosphate-buffered saline), 28 μl of 0.3 M EDTA and 12 μl of phenylmethylsulfonlylfluoride (PMSF; in 95% alcohol), followed by 30 μl of newborn calf serum 2 min later. Samples were immediately frozen at −70 °C. After 4 h of incubation the remaining 1500 μl was similarly treated and frozen in 500-μl aliquots.

Without any cytokine digestion, this sample dilution would lead to concentrations of cytokine of 806.45 pg/ml IL-8 and 1832.84 pg/ml TNFα. To test the cytokine recovery of the assay in this environment, triplicate samples from digestion of IL-8 had an IL-8 spike added to increase the IL-8 concentration by 1000 pg/ml before reassay (Fig. 1).

2.2. Sputum

In order to assess the influence of sputum on enzymatic digestion of cytokines, fresh sputum from a boy with end-stage CF lung disease was homogenised in buffer (1 g/ml) and assayed for IL-8 concentration following incubation with Creon Forte and with control buffer.

2.3. Cytokine transit through the Wistar rat gut

A total of 15 male Wistar rats (200 g) were divided into three groups of five rats. All 15 rats were given a 1-ml gavage of either phosphate-buffered saline (PBS), interleukin-8 (3000 pg) in PBS or interleukin-8 (3000 pg) and TNFα (200 pg) in PBS. These were weight-for-weight dosages equivalent to a 60-kg patient swallowing 30 g of sputum containing typical amounts of IL-8 [12] and TNFα [13]. The rats were caged above wire mesh and stool collection commenced. They were given a 1-ml gavage of Indian ink (Pelikan) 6 h later, and stool collection continued at 6-h intervals until faecal pellets became black. Faecal pellets containing ink were not included in the collection for homogenisation. Faecal samples were immediately frozen at −20 °C. Frozen faecal samples were powdered using a mortar and pestle, homogenised in five times their weight of phosphate-buffered saline and centrifuged at 20 000×g for 15 min. Supernatant was frozen at −70 °C and subsequently analysed for IL-8 and TNF concentrations, using a protocol previously validated for use of the Quantikine kits with human samples [5].

2.4. Faecal supernatant cytokine assays

All faecally derived samples were prediluted 1:1 in newborn calf serum, and any further dilution was made using calf serum as the diluent. Cytokine assays were performed using TNFα and IL-8 Quantikine kits (R&D Systems Europe, Abingdon) according to the manufacturer's instructions for cell culture samples.

To confirm assay linearity for the faecally derived samples, an additional four-fold dilution in newborn calf serum was analysed for each sample. The assay was accepted as being linear for each sample if the cytokine concentration calculated from each dilution was within 10% of the mean result for both dilutions; accordingly, this mean aliquot cytokine concentration was used to calculate the faecal cytokine concentration. With this protocol in the IL-8 assay, using human faecal supernatants, the intra-assay coefficient of variation was 3.46% ($n=7$), the inter-assay coefficient of variation was 6.85% ($n=5$) and mean (S.D.) spike recovery was 92.12% (12.48%) ($n=8$). For the TNFα assay these figures were 4.57% ($n=10$), 6.92% ($n=2$) and 91.48% (5.57%) ($n=8$), respectively.

Duplicates of supernatants from faeces of four of the five rats dosed with only phosphate-buffered saline were spiked with IL-8 to 500 pg/ml to check that the human faeces assay protocol was valid in Wistar rat faeces.

2.5. Histology

When faecal specimen collection was complete, the rats were euthanased with a lethal (1–3 ml) intraperitoneal dose of phenobarbitone sodium (325 mg/ml; Lethobarb,Virbac, Sydney, NSW). Their gastrointestinal tracts were dissected and representative samples of stomach, duodenum, ileum and colon were taken and processed for histological examination. These were examined blind for the presence of intestinal inflammation.

Blinding was achieved in the histology processing laboratory by technical staff who played no part in examination of the histology. Cassettes containing the

rat tissue were labelled with the rat serial number and the site of origin of the tissue. Haematoxylin- and eosin-stained slides were labelled with a random serial number. The randomisation key was recorded on a log sheet and placed in a sealed envelope. This was not opened until after the slides had been examined.

All tissue on each slide was examined at 400× magnification for the presence of neutrophil polymorphs. Segmented polymorphs that did not have demonstrably eosinophilic granules were classified as neutrophils. In order to identify all possible foci of acute inflammation, all tissue neutrophils observed were recorded. A focus of inflammation was defined as 'at least three neutrophils in one high-power (400×) field.'

2.6. Statistical methods

Analysis of variance and paired *t*-tests were used to test differences between groups where appropriate.

2.7. Ethical approval

The study was approved by the University of Queensland animal experimentation ethics committee and the ethics committee of The Royal Children's Hospital, Brisbane.

3. Results

3.1. Enzymatic cytokine digestion in vitro

3.1.1. Interleukin-8 assay

No IL-8 immunoreactivity was detected in any of the triplicate tubes loaded with both enzyme and cytokine, at incubation of either 4 or 1 h, at either pH 8 or pH 4.5. Mean IL-8 concentrations in the buffer samples were: 603 pg/ml at 1 h and 853 pg/ml at 4 h, an apparent rise ($t=6.05$, $P=0.004$), at pH 8.0; and 587 pg/ml at 1 h and 514 pg/ml at 4 h, no significant change ($t=2.31$, $P=0.08$), at pH 4.5.

3.1.2. Spiked IL-8 digestion samples

The mean IL-8 concentration for the nine samples from the IL-8 digestion at pH 8 that were subsequently spiked to 1000 pg/ml, using the assay 2000 pg/ml IL-8 standard as spiking solution, was 858.77 pg/ml. Mean IL-8 concentrations were 873.68, 864.54 and 838.09 pg/ml for Creon Forte, Pancrease and Cotazym co-incubated and spiked samples, respectively.

3.2. TNFα assay results

TNFα was measurable in all TNFα samples subjected to digestion at pH 4.5 and pH 8.0, with a progressive reduction in TNFα concentration between 1 and 4 h of enzyme co-incubation, whilst the control samples without enzyme maintained their high cytokine concentrations. Samples that were not inoculated with cytokine had no TNFα immunoreactivity. Results are shown in Table 1.

Table 1
TNFα concentrations following in vitro digestion

Preparation	Tryptic activity (kU/l)	TNFα concentration (pg/ml)[a]			
		pH 8		pH 4.5	
		1 h	4 h	1 h	4 h
Creon Forte	4.3	1066.20	132.82	>2000	986.36
		1027.67	124.14	>2000	1000.54
		955.16	125.67	>2000	1024.05
Pancrease	17.7	797.61	118.56	31.59	7.38[b]
		933.60	115.26	29.93	5.12[b]
		929.73	128.72	35.00	8.31[b]
Cotazym S	15.1	943.71	117.29	197.67	16.22
		908.74	140.43	35.00	12.77[b]
		961.80	136.61	39.56	13.64[b]
Buffer	<0.05	3470.90	3822.90	5614.91	5244.66
		2711.32	3542.54	5405.56	4807.61
		2659.56	3592.49	5779.02	5779.02

[a] Expected concentration 1833 pg/ml.
[b] Calculated by interpolation.

3.3. Sputum sample

Interleukin-8 recovered from the CF sputum incubated with buffer was 130 150 pg/g, whilst 34 680 pg/g was recovered from the sample incubated with Creon Forte at pH 8.0. Neither the digested nor the undigested sputum samples contained assayable concentrations of TNFα.

3.4. Cytokine transit through the Wistar rat gut

Mean stool weight collected was 2.67 g for rats dosed with PBS alone, 2.53 g for rats dosed with 3000 pg IL-8 in PBS, and 2.98 g for rats dosed with 3000 pg IL-8 and 200 pg TNFα in PBS. These stool weights were not significantly different ($F=1.51$, $P=0.26$ by ANOVA).

No TNFα immunoreactivity was detected in the faecal samples of the five rats that were given TNFα and IL-8, nor in the five control rats that received phosphate-buffered saline.

No significant IL-8 immunoreactivity was detected in any experimental or control rats. Of the four faecal samples that were spiked to 500 pg/ml, all had assayable quantities of IL-8 (Table 2), with a mean IL-8 concentration was 773 pg/ml. Table 2 also shows the percentage variation in the cytokine concentration determined from two dilutions of the same sample. These show that the assay was linear for these samples.

Table 2
[IL-8] in control aliquots spiked to 500 pg/ml

Rat number	IL-8 concentration (pg/ml)	Dilution difference (%)
7	745	4.2
8	809	3.5
9	733	5.5
10	807	2.1

3.5. Histology

Of the four sampling sites for the 15 rats, a single slide demonstrated a focus of inflammation. This occurred in stomach tissue just distal to the gastro-oesophageal junction, in a rat who had been dosed with 3000 pg of IL-8. This rat also had two isolated neutrophils in the distal small bowel, and two isolated neutrophils in the colon. Two other rats dosed with IL-8 alone had tissue neutrophils identified, one with three isolated neutrophils in the colon and the other with a single neutrophil in the proximal small bowel.

A single neutrophil was observed in the colon of one rat dosed with only phosphate-buffered saline. Four of five rats dosed with both IL-8 and TNFα were noted to have solitary tissue neutrophils, but no focus of inflammation. One of these rats had solitary neutrophils in stomach, distal small bowel and colon, one in distal small bowel and colon, and one rat each in proximal and distal small bowel.

4. Discussion

With in vitro digestion, the presence of assayable quantities of TNFα in the samples for all three enzyme preparations at both time points allows us to conclude that there had been a progressive decrease in TNFα concentration with time in these samples. That this decrease was smallest in the preparation with the lowest tryptic activity measured suggests that enzymatic activity is responsible for the decrease. It might be argued that the failure to detect IL-8 in the digestion experiment was the result of enzymatic interference with the assay rather than cytokine digestion, but the subsequent recovery of an average 85% of an IL-8 spike after the addition of protease inhibitors makes this unlikely. We are led to conclude that both IL-8 and TNFα are efficiently digested by all three tested pancreatic enzyme preparations both at pH 4.5 and pH 8. The four-fold reduction in sputum IL-8 concentration measured on incubation with Creon Forte under circumstances that abolished IL-8 immunoreactivity in aqueous samples suggests that sputum may provide a degree of protection for the chemokine against pancreatic enzymes.

Two technical points in these assays deserve comment. Firstly, we have observed a significant reduction in IL-8 recovery in buffer incubation at 1 h and not at 4 h, and secondly, the concentrations of TNFα calculated following buffer incubation at both pH 4.5 and pH 8 show concentrations of over two-fold the concentration administered. We suspect that these perturbations are due to the sensitivity of the ELISA assays to minor changes in the chemical composition of the assay wells. This would be in accord with the non-linear assay characteristics demonstrated in our previous work on unvalidated assays in human faecal homogenate. Despite these variations from ideal behaviour in the control samples, the contrast with the changes observed in the enzyme-incubated samples is marked, and there remains convincing evidence of enzyme digestion in those wells.

In the rat-gut cytokine transit experiment, it was necessary to have a significant interval between cytokine and Indian ink administration so that the cytokines were not adsorbed onto the carbon particles of the ink. The cytokine dilution resulting from this interval exposed the study to the risk that cytokine was diluted beyond the detection limit of the assay. The maximum weight of rat faecal pellets was 3.44 g. If all of the 3000 pg of IL-8 and 200 pg of TNFα administered to the rat passed through the gut, cytokine concentration in the aliquots would be 145 pg IL-8/ml and 9.6 pg TNFα/ml. These concentrations of IL-8 and TNFα were 10- and two-fold the assay detection limit, respectively. It is safe to conclude that the Wistar rat destroys at least half of the administered dose of TNFα and at least 90% of the administered dose of IL-8 during passage through its intestine. This is consistent with the efficient digestion of these cytokines in the pancreatic sufficient mammal. Whilst faecal pellets were collected at 6-h intervals and would have been at room temperature for this time, we have assumed that little cytokine digestion occurred because of the dry nature of the pellets.

This efficient digestion is consistent with the lack of an intestinal inflammatory response. The sole instance of intestinal inflammation in one rat stomach, just distal to the oesophago-gastric junction, could be a result of the administration of IL-8 to the stomach. However, the fact that only one rat was thus affected is more consistent with inflammation having been caused by the process of passing the gavage needle through the gastro-oesophageal junction. This study was designed not to miss a subtle acute inflammatory focus. The tissue neutrophils observed were not sufficiently numerous to satisfy the study criteria for a focus of inflammation, but they could signify mild acute inflammation. All but one of the slides containing isolated neutrophils were in rats that were gavaged with a dose of IL-8, despite the blinding of the histological assessor to the origin of the slides. Because of the possibility of misclassifying the morphology of an individual polymorph, we had required three neutrophils per high-powered field to conclude that a focus of inflammation was present. Whilst this

criterion was arbitrary, it was set at the stage of study design and should be adhered to. In making a post-hoc assessment of these data, we should consider the proportion of the rat gut that was examined. We estimate that a total of 8 cm×5 μm of tissue was examined for each rat, and that the length of each rat gut was in excess of 40 cm. With this proportion of tissue examination, we do not believe that our data support the existence of even the most subtle of inflammatory responses to gavaged cytokine.

We conclude that the bulk of swallowed IL-8 and TNFα is digested in pancreatic sufficient animals, and that modern pancreatic enzyme replacement therapy is also effective at digesting these cytokines, even at the sub-optimal pH likely to be encountered in the CF small intestine. Whilst cytokines in sputum are also subject to digestion, they may enjoy a degree of protection from digestion by the physical nature of the sputum. Any such protection is likely to be influenced by any variant gastric motor function in cystic fibrosis [14–16], and further work could explore these issues. Small amounts of swallowed cytokine may gain access to the intestinal lamina propria and remain physiologically active, but we have not found sufficient evidence to suggest that doses of cytokines swallowed by cystic fibrosis patients could be measured as an index of pulmonary inflammation, or act as a cause of intestinal inflammation. Only if whole gut transit time were reduced to 1 or 2 h would we expect to be able to assay any sputum-derived cytokines in faeces, a situation that would occur in whole-gut lavage studies. Faecal cytokines that we have previously described [3] are unlikely to originate from sputum and we believe that they are a result of artefact. Cytokines in whole-gut lavage fluid may result from pulmonary or intestinal inflammation or artefact. Smyth et al. have recently ascribed the inflammatory markers they detected in whole-gut lavage fluid to constitutional intestinal inflammation in cystic fibrosis [4]; however, their assay protocols were unvalidated and high IL-8 concentrations in their study may well be overestimates of the true concentration [17]. This makes it likely that they have been detecting IL-8 immunoreactivity resulting from the intestinal transit of pulmonary-derived cytokines. Even if whole-gut lavage cytokine concentrations could be measured reproducibly, the invasive nature of whole-gut lavage in children would be an obstacle to its use as a proxy measure of pulmonary inflammatory activity.

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