ORIGINAL ARTICLE

Upregulated eotaxin expression and T cell infiltration in the basal and papillary epithelium in cows' milk associated reflux oesophagitis

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Background: Cows' milk sensitive reflux oesophagitis is an emerging clinical entity in children, normally indistinguishable from primary gastro-oesophageal reflux (GOR) apart from the response to dietary antigen exclusion. It is conjectural whether a tendency towards mucosal eosinophilia distinguishes this group from primary GOR.

Aims: To determine whether there may be differences in the mucosal lesion within the oesophagus in those children with reflux in association with cows' milk induced small bowel pathology, particularly in relation to the eosinophil chemokine eotaxin.

Methods: A total of 29 children underwent endoscopic assessment, including nine with cows' milk sensitive enteropathy (CMSE) and associated GOR, seven histologically normal controls, six with primary GOR, and seven disease controls. Oesophageal biopsy specimens were examined immunohistochemically for the chemokines eotaxin and MCP-2, and T cell lineage and activation markers.

Results: Strong upregulation of eotaxin expression, limited to basal and papillary epithelium, occurred in all CMSE patients. By contrast, weak expression was seen in a minority of controls and in 50% of primary GOR patients. Infiltration of CD3, CD4, and CD8 lymphocytes occurred in similar distribution in CMSE patients, significantly increased above controls. Significant upregulation of activation markers (CD25, HLA-DR) was also seen in the CMSE group within basal and papillary epithelium compared to controls and primary GOR.

Conclusion: Basal and papillary epithelial eotaxin expression, with focal lymphocyte activation, was seen in infants with CMSE associated GOR. This preliminary study provides early evidence to suggest a pathogenesis distinct from primary GOR, in which specific recruitment of T cells and eosinophils may contribute to oesophageal dysmotility.

Gastro-oesophageal reflux (GOR) is a common phenomenon in infancy, which may become pathological and result in a wide range of clinical manifestations and significant morbidity.[1] There is lack of agreement regarding the definition and significance of one of its potentially most important complications, oesophagitis, as assessed by standard diagnostic techniques, including endoscopy and biopsy.[2][3] In particular, these techniques do not reliably distinguish between primary reflux oesophagitis (PRO) and the emerging clinical entity of cows' milk associated reflux oesophagitis. This variant of cows' milk allergy appears to be a particularly common manifestation in infancy, with symptoms indistinguishable from primary gastro-oesophageal reflux but settling on an exclusion diet.[4] Some differentiation from primary reflux has been suggested on the basis of oesophageal pH testing pattern and β lactoglobulin antibody response.[4][5] There is recent evidence that upper gastrointestinal symptoms are becoming a more common presentation of infant food allergy within the developed world, and in fact may be induced to a variety of antigens in addition to cows' milk.[6][7] A defect in oral tolerance for low doses of antigen has been postulated as the underlying cause.[8][9]

Oesophageal mucosal eosinophilia has been described in both suspected cows' milk associated[6] and primary reflux oesophagitis,[10] as well as in other conditions such as primary eosinophilic oesophagitis.[11] The clinical significance of eosinophils and their role in the pathogenesis of mucosal injury is poorly understood and the subject of recent debate.[2][3][12] In addition to dietary exclusion of cows' milk,[4][6] oral steroids can induce remission of symptoms with decreased mucosal eosinophilia,[6][11] suggesting a pathoaetiological role for eosinophils. In addition to eosinophils, epithelial T lymphocytes, known as cells with irregular nuclear contours (CINC), have also been implicated as markers of reflux oesophagitis.[13][14] In adults such cells are of memory phenotype and display activation markers,[15] although little is known of their paediatric equivalents .

We have studied a group of patients with clinically and histologically proven cows' milk sensitive enteropathy (CMSE) and associated reflux symptoms. We examined the relation between cows' milk induced small bowel pathology, GOR, and the pattern of the oesophageal mucosal immunological response. A variety of monoclonals were used to examine the oesophageal mucosa, including eotaxin, a recently described eosinophil specific chemokine,[16] markers of T cell lineage (CD3, CD4, CD8) and activation (CD25, HLADR), cell proliferation markers (Ki67), and architectural markers of basement membrane (tenascin). Current murine evidence suggests that mucosal eotaxin expression may be critical in the allergic response to dietary antigen.[17] Our aim was to determine whether there is a specific immunological lesion within the oesophagus in cows' milk sensitive reflux that may be distinct from primary GOR.

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Abbreviations: CMSE, cows' milk sensitive enteropathy; GOR, gastro-oesophageal reflux

Table 1 Patient characteristics

Group	Sex	Age (mth)	Symptoms	Treament (prestudy)	pH reflux index (%)	pH pattern of tracing
(1) CMSE						
1	M	36	V, D, FTT	nil†	12.7	np
2	F	3	V, D, FTT, colic	nil	26	np
3	F	19	V, D, FTT	nil	24.5	np
4	M	12	V, poor feeding, FTT	cis, ran	7.6	np
5	F	5	V, D, food refusal, FTT	nil	23.6	np
6	M	11	V, D, FTT	nil	6.2	np
7	M	10	V, D, FTT	nil	15.3	np
8	M	7	V, poor feeding	nil	8.4	np
9	F	88	V, haematemesis, abdominal pain	nil	15.4	np
(2) Controls						
1	F	3	V	nil	7.5	np
2	M	33	V, D, *	cis, ran	9	np
3	M	8	Disturbed feeding, V	nil	6.3	np
4	F	140	V, abdominal pain	nil	0	np
5	M	14	D, V	nil	19.8	np
6	M	49	Non-specific abdominal pain	nil	2.1	np
7	M	141	Non-specific abdominal pain	cis, ran	5	np
(3) PRO						
1	M	15	V, FTT	cis, ran	17.3	np
2	F	22	V, FTT	nil	3.2	np
3	F	81	V, FTT	cis, ran	64.7	np
4	M	24	Disturbed feeding, V, FTT	nil	9.6	np
5	M	5	V, haematemesis, melaena	nil	7.5	np
6	M	14	V, FTT	cis, ran	87	np
(4) DC						
1	F	34	D, abdominal distension, FTT	nil	na	na
2	M	4	D, generalised oedema, FTT, *	nil	55.4	np
3	M	43	Short stature, D	nil	na	na
4	M	95	D, rectal bleeding	nil	na	na
5	F	57	Abdominal pain, V	nil	12	np
6	M	85	D, V	nil	0.7	np
7	M	184	Abdominal pain, rectal bleeding	nil	5	np
Total (n=29)						

*Wheeze; †egg/milk/wheat free diet.
V, vomiting; D, diarrhoea; FTT, failure to thrive; cis, cisapride; ran, ranitidine; np, non-phasic; na, not available.

METHODS
Patients
Twenty nine children (19 boys, 10 girls, age range 3–184 months), were enrolled consecutively following referral to our department for investigation and management of clinical symptoms suggestive of reflux oesophagitis and/or enteropathy. All were evaluated by endoscopy with oesophageal and small bowel mucosal biopsies. The protocol and study design were approved by the local research ethics committee of the Royal Free and University College School of Medicine; informed parental consent was obtained in all cases. Four subgroups of children were defined as follows.

Group 1 (n = 9), cows' milk sensitive enteropathy (CMSE): children initially referred with a history of vomiting, poor weight gain, and/or loose stools with associated symptoms suggestive of GOR. Table 1 gives clinical details; table 2 gives results of 24 hour pH testing. The standard oesophageal histological features of oesophagitis using ESPGHAN criteria were noted,[18] and small bowel biopsy in each child showed enteropathy characteristic of CMSE.[9] All showed clear symptomatic improvement on cows' milk exclusion (5/9 requiring an amino acid formula), with recurrence of symptoms on open challenge. Systematic open challenges were performed on a day case inpatient basis on a careful increasing dose regime administered by a clinical nurse specialist and under the care of a paediatric member of the medical team. Response was identified according to our normal protocol as either an acute type 1 response of cutaneous or respiratory nature or a delayed type 3 response after ingestion for up to seven days of a gastrointestinal nature—

including recurrence of GOR type symptoms. A degree of subjectivity was inevitable given that these were not double blind challenges.

Group 2 (n = 7), controls: those children in whom investigation showed both normal oesophageal and small bowel histology.

Group 3 (n = 6), with primary reflux oesophagitis (PRO): determined by chronic symptom history, pH study, and standard oesophageal histological features of oesophagitis using ESPGHAN criteria.[18]

Group 4 (n = 7), disease controls (DC): primary inflammation is elsewhere in the GI tract (two cases of coeliac disease, one case of congenital disorder of glycosylation and enteropathy, one case of intestinal giardiasis, two cases of inflammatory bowel disease affecting the large bowel, and one case of *Helicobacter pyloric* gastritis).

Other routine investigations, performed as part of the diagnostic workup as clinically indicated, included haemoglobin, eosinophil count, serum IgE, and other immunoglobulins and did not differ between the groups (data not shown).

Endoscopy and collection of mucosal specimens
Upper gastrointestinal endoscopy was performed in all patients under general anaesthesia. Oesophageal mucosal grasp biopsies were performed using paediatric endoscopes, Olympus N30 or XQ230 at 2–3 cm above the gastro-oesophageal junction, or at the site of any macroscopic abnormalities. In addition serial biopsy specimens were taken from the gastric antrum and the fourth part of the duodenum. Biopsy specimens were collected in formalin for routine

Table 2 Twenty four hour pH studies

Groups	Completed studies	Median reflux index (%)	Reflux index range	Abnormal studies (%)	Pattern of pH tracing
CMSE	9	15.3	7.6–26	89	Non-phasic
Control	7	6.3	0–19.8	43	Non-phasic
PRO	6	13.5	3.2–87	67	Non-phasic
DC	4*	8.5	0.7–55.4	50	Non-phasic

3 pH study data unavailable.

Table 3 Monoclonal antibodies used

Antibody	Specificity	Manufacturer	Working dilution
CD3	T lymphocytes	DAKO	1:40
CD4	T helper/inducer lymphocytes	DAKO	1:10
CD8	T cytotoxic lymphocytes	DAKO	1:50
CD25	Interleukin 2 receptor, activated T cells	DAKO	1:40
HLA-DR	MHC type II receptor activated T cells	DAKO	1:40
Ki67	Proliferating cell nuclei	DAKO	1:25
CD138	Syndecan-1, matrix glycoprotein	Serotec Ltd	1:100
6H9	Eotaxin	See text	1:200
2D5	Monocyte chemotactic peptide 2	See text	1:100
Tenascin	Basement membrane component	See text	1:2000

histology, or snap frozen in liquid nitrogen for immunohisto-chemistry.

Assessment of oesophageal tissue

Light microscopic assessment was performed, using standard processing techniques on formalin fixed tissue. Haematoxylin and eosin (H&E) stained specimens were evaluated by an experienced histopathologist (PK), completely blinded to the clinical history and endoscopic findings, for the following: (1) grading of severity of reflux oesophagitis according to ESPGHAN criteria[18]; (2) scoring for the number of intraepithelial eosinophils; and (3) scoring for the number of intraepithelial lymphocytes (both scored as none, <10, or >10 per high power field × 250; modified from Cucchiara and colleagues[19]).

Immunohistochemistry

Frozen biopsy specimens were orientated and mounted, serial cryostat sections were cut at 4 µm and fixed in acetone, staining with the alkaline phosphatase antialkaline phosphatase (APAAP) technique, and then stained with a range of monoclonal antibodies (mabs) for cell lineage and activation markers (table 3). Commercially obtained antibodies were supplied by Dako, High Wycombe, UK and Serotec Ltd, Oxford, UK. Monoclonals 6H9 and 2D5 against eotaxin and MCP-2[20] were kindly supplied by Dr Charles Mackay, LeukoSite Inc., Cambridge, Massachusetts, USA. Neither antibody shows cross reactivity with the other, or chemokines including MCP-1, MCP-3, RANTES, MIP-1β, IL-8, GRO-α, or NAP-2.

Evaluation of staining

This was carried out by two observers (AB and SHM) independently and both were blinded to the clinical grouping of the specimens, examining slides using standard light microscopy at high power field (HPF) magnification (objective lens ×40). Overall correlation between observers was high (>95% concordance); slides which had been scored differently were reviewed by both observers jointly, with a consensus decision reached. Assessments were made of the following:

- Location of staining within the oesophageal epithelial compartments: SE, superficial epithelium; PE, prickle epithelium; BE, basal epithelium; PAP, papillae.

- Staining score, determined by the density of positive staining cells over at least five high power fields, or by the strength of epithelial immunoreactivity as appropriate, within each compartment. Scores were graded 0–4 (0 = no positive cells/no epithelial staining; 1 = occasional positive cell (less than 1 cell/high power field)/weak epithelial staining; 2 = 2–5 cells per high power field/moderate epithelial staining; 3 = 6–10 cells per high power field/strong epithelial staining; 4 = >10 cells per high power field/very strong epithelial staining).

- Basal cell proliferation was assessed by counting the cell depth of Ki67 positive cells in the basal epithelium (that is, cell depth number).

Twenty four hour oesophageal pH monitoring

pH monitoring was performed on the same admission as the endoscopic assessment. The probe was positioned in the oesophagus at the time of endoscopy or placement was confirmed by chest *x* ray before measurement began. Standardisation of techniques and measurements was in accordance with ESPGHAN guidelines.[21] An abnormal pH study was defined as a reflux index greater than 10% in those under 1 year of age and greater than 6% in those over 1 year of age. In addition the graphic tracing was examined for evidence of abnormalities in the pattern related to feeding, posture, or symptoms, in particular looking for "phasic" changes suggested by Cavataio *et al* as predictive of CMP associated GOR; none were observed.[5]

Statistical analysis

For descriptive purposes, the full staining scores were presented graphically; these were also categorised into those with "negative" (score 0–1) and "positive" (score >1) staining scores, for analysis of the proportion (%) of patients with "positive" staining scores. These were cross tabulated by the patient groups: normal control patients were compared with each of the disease groups. Fisher's exact test (two tailed) was used to assess the statistical significance of differences between the groups.

The relations of potential confounding factors of sex and age with staining scores were investigated for significance using Fisher's exact test and analysis of variance respectively.

RESULTS

Tables 1 and 2 outline patient group characteristics. There was no statistically significant association of those variables with any of the staining scores. A high mean reflux index on pH testing was found in 89% of the CMSE group compared to 43% of controls (table 2). The pattern of pH tracing was "non-phasic"[5] in all patients studied, including those in the CMSE group.

Oesophageal histological assessment

The proportion of patients with abnormal histology was significantly different in the CMSE and primary reflux oesophagitis groups compared to controls (CMSE group: 5/9 patients and primary reflux group: 6/6; compared to controls: 0/7, p < 0.05). This was graded mild (grade 2 or less) in all except one case in the primary reflux group, which was grade 4. The proportion of patients with increased intraepithelial eosinophils and lymphocytes respectively were as follows: CMSE: 1/9 (11%) and 5/9 (56%); controls: 0/7 (0%) and 1/7 (14%); primary reflux oesophagitis: 3/6 (50%) and 3/6 (50%); and disease controls: 1/7 (14%) and 2/7 (29%). These differences did not reach statistical significance.

### Oesophageal immunohistochemistry

Expression of chemokines and class II MHC within the epithelium

The intensity of eotaxin expression was significantly increased in the basal epithelium in group 1 (CMSE group) compared to group 2 (controls) (p = 0.001) (figs 1 and 5). The proportion with positive eotaxin staining when compared separately in the basal epithelium and papillary epithelium was significantly higher in both areas compared to group 2 (controls) and group 3 (primary GOR): basal epithelium 8/8 versus 3/7 controls and 3/6 with PRO (p < 0.05); papillary epithelium 9/9 versus 1/5 controls and 3/6 with PRO (p < 0.05) (fig 2). By contrast there were no significant differences in basal epithelial expression between the primary reflux oesophagitis, disease control, and control groups. Where subepithelial lamina propria tissue was included within the biopsies, strong eotaxin expression could often be seen on vascular endothelium. However, this was not restricted to the oesophagitis groups, and was found in several controls.

HLA-DR expression was detected in the basal epithelium in all (9/9) patients with CMSE compared to 2/5 controls (p < 0.03) as well as 4/6 in both the primary reflux oesophagitis and disease control groups (figs 3 and 5). Strong expression (grade 3 or 4) was seen in 5/9 with CMSE, 1/6 with primary reflux oesophagitis, and 3/6 disease controls.

Lymphocyte infiltrate

The CMSE group showed significant excess infiltration (score >1) of CD4 cells within the basal epithelium in 6/9 patients compared to 0 controls (p < 0.01), 3/6 with primary reflux oesophagitis, and 2/6 disease controls (figs 3, 4, and 5). These lymphocytes showed expression of the activation marker CD25 in 4/9 CMSE cases, 0 controls, 1/6 with primary reflux oesophagitis, and 0/6 disease controls (p < 0.05) and were frequently HLA-DR positive (figs 3 and 5). No statistically significant differences were observed in these markers between the other groups. Within the papillae, increased numbers of CD3, CD4, and CD25$^+$ cells were found in the CMSE group compared to controls, in whom CD25 expression was not detected. CD3 cell infiltration was seen in all the CMSE group compared to 3/7 controls (p < 0.05), with no significant differences seen between the other groups and controls. CD4 cell infiltration showed similar distribution within the groups (fig 4).
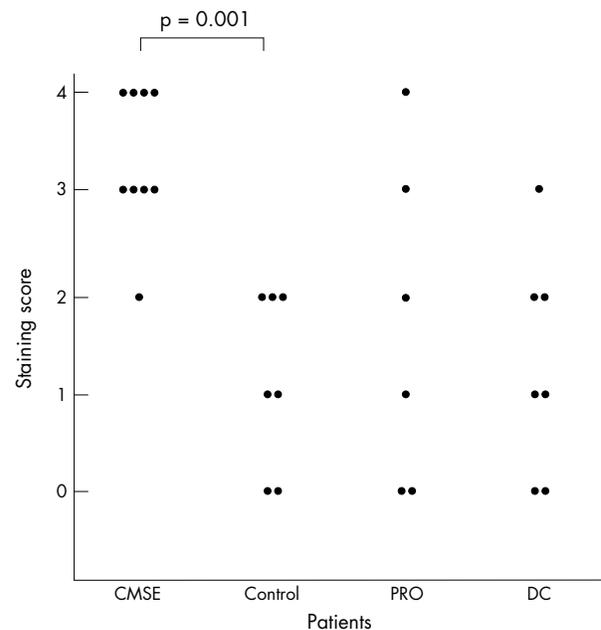
Figure 1 Eotaxin expression within the basal oesophageal epithelium in CMSE, histologically normal controls, primary reflux oesophagitis (PRO), and disease control (DC) groups. Epithelial staining, graded 0–4, as determined by intensity of staining (see Methods).
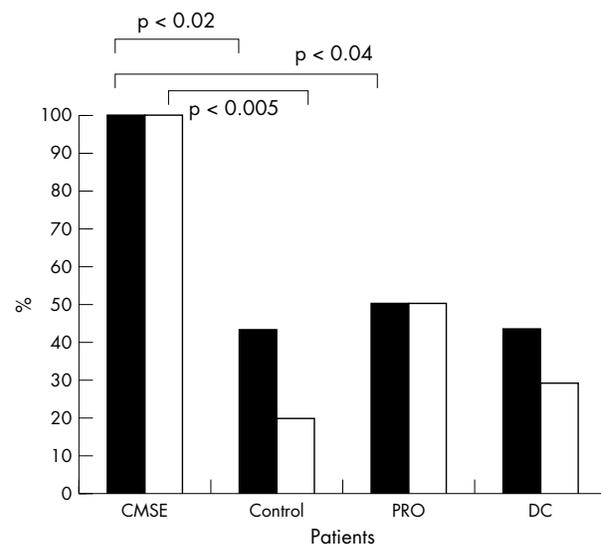

Figure 2 Pattern of eotaxin expression; proportion of patients with "positive staining" (staining score >1) of basal (closed bars) and papillary (open bars) oesophageal epithelium. Patient groups as in fig 1.

The primary oesophageal reflux group were the only others apart from the CMSE group to show papillary CD25$^+$ cells, detected in 4/6 cases.

Epithelial proliferation and basement membrane composition

Examination of epithelial markers showed a characteristic distribution of the proteoglycan syndecan-1 in all groups, with strong staining in the basal epithelium and papillae, diminishing strikingly towards the superficial epithelium. This was not altered in oesophagitis. The proliferation marker Ki67 was uniformly distributed throughout the basal and papillary epithelium in all cases and in all groups. Thus
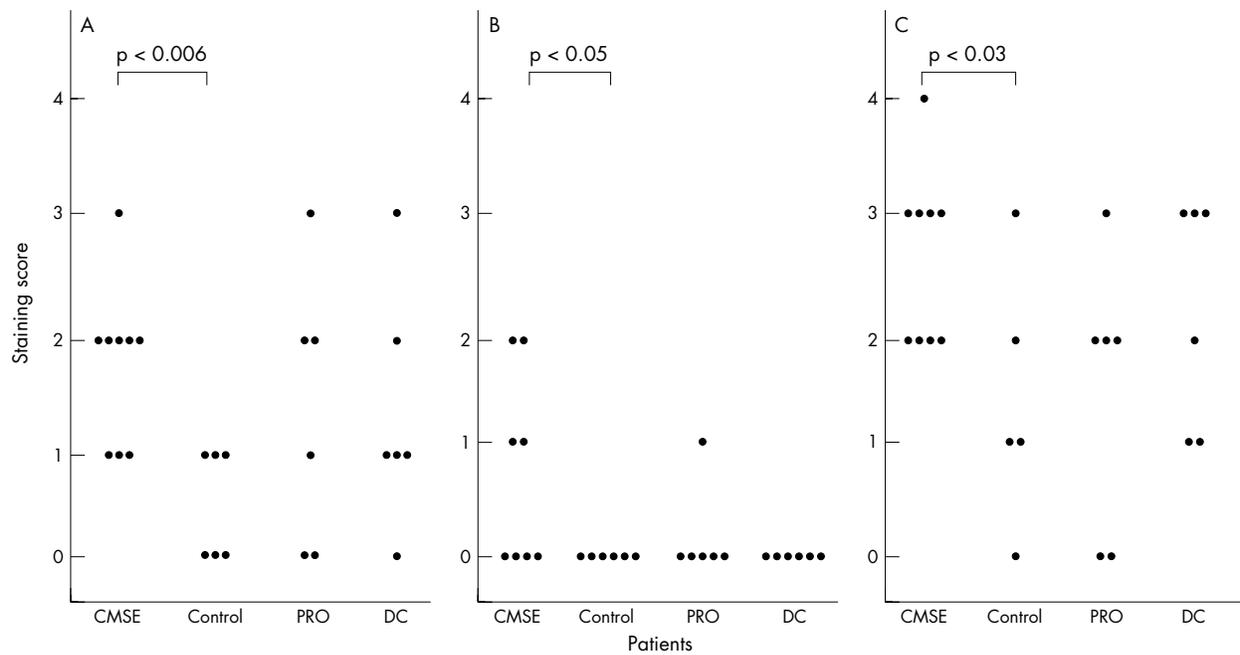
Figure 3 Density of (A) CD4 and (B) CD25 cell infiltrates with (C) intensity of HLA-DR expression within basal oesophageal epithelium in the comparative groups. Symbols as in fig 1.
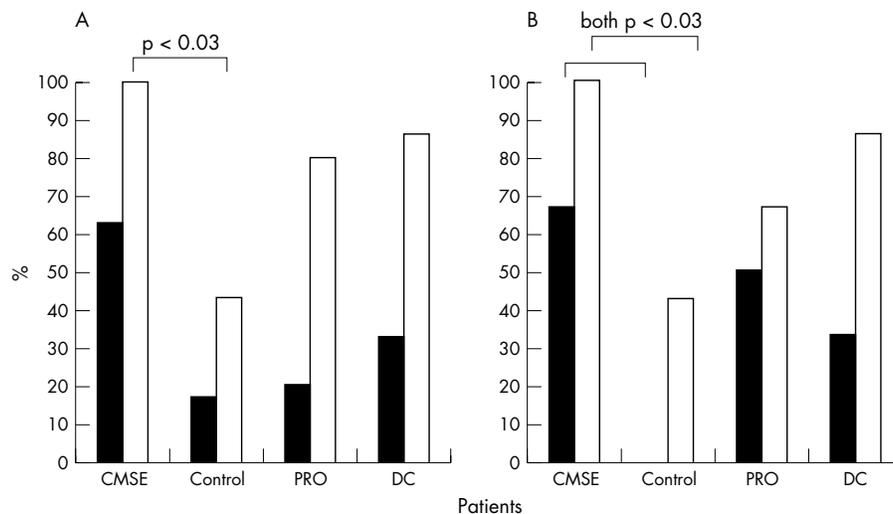
Figure 4 Proportion of patients with cell density score >1 for (A) CD3 and (B) CD4 cells within the basal (closed bars) and papillary (open bars) oesophageal epithelium in the comparative groups.

increased papillary length concorded with increased total numbers of proliferating Ki67^{+} cells, and was seen in both CMSE associated and primary reflux oesophagitis. However there was no overall difference between the groups in the numbers of Ki67^{+} cells in the basal epithelium. The basement membrane component tenascin was strongly expressed in both cases and controls within the basement membrane subjacent to the basal epithelium, extending into the papillae along their full length. However, staining intensity and basement membrane thickness did not differ between groups.

DISCUSSION

We have identified differences between cows' milk induced oesophageal reflux and primary reflux oesophagitis, with the former showing increased expression of eotaxin localised to the basal and papillary epithelium, which also showed upregulated HLA-DR. We accept the small size of the patient

groups which may have underestimated the potential difference, for instance, between the primary reflux group and the control group—nevertheless this makes the differences found between the CMSE and the control groups potentially more important. It is interesting that the acid reflux was equally severe in the PRO group and the CMRO group. This supports recent murine evidence that eotaxin may play an important role in food allergic responses.[17]

In addition to the focal eotaxin expression, there was a similar distribution of activated T cells, within and subjacent to the basal and papillary epithelium. Although there are no published data showing expression of eotaxin in the human oesophagus, this has been shown in the normal small and large bowel and other tissues.[16] Eotaxin deficient mice show reduced intestinal eosinophils,[22] providing evidence for its constitutive expression and suggesting that eotaxin expression in our controls reflects normal regulation of eosinophil
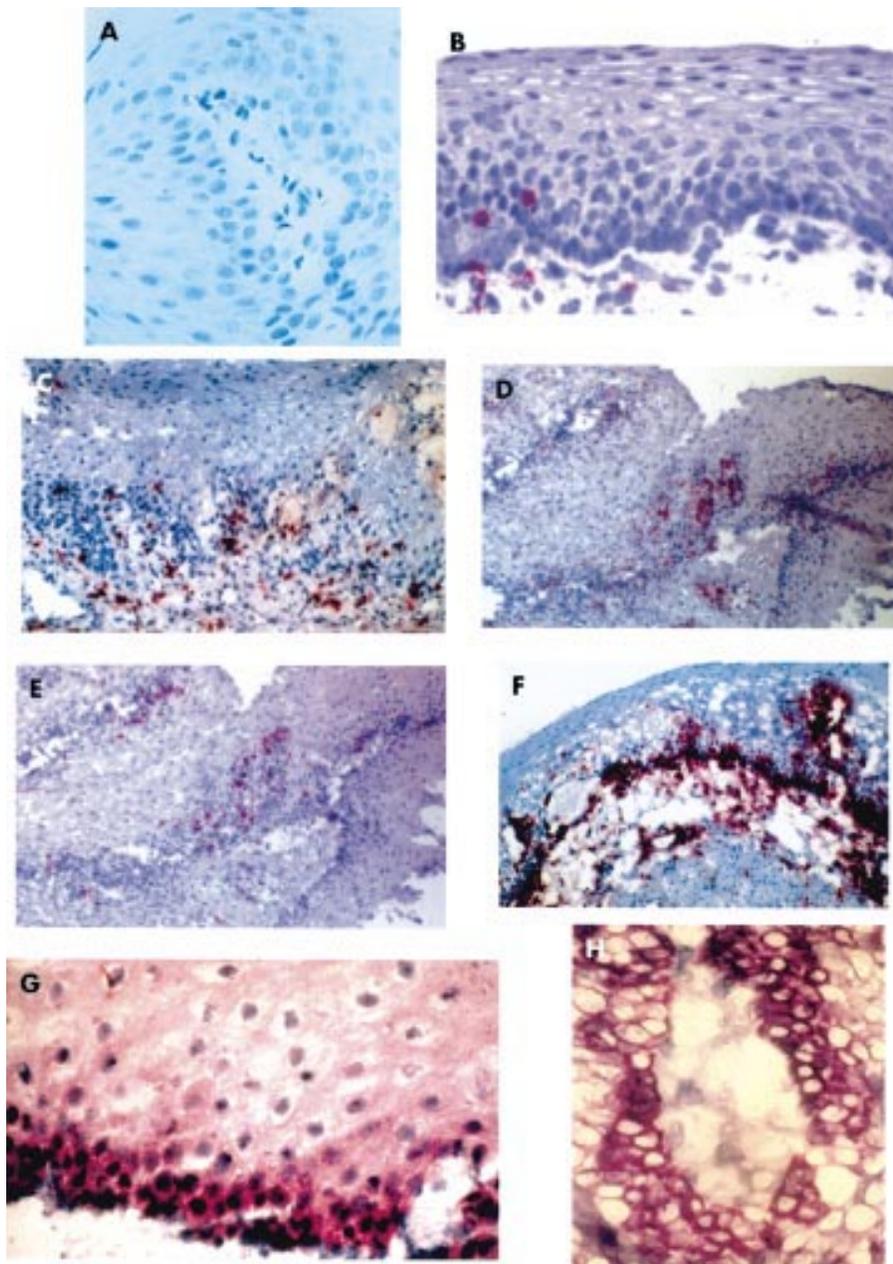
Figure 5 Histological and immunohistological findings in CMSE associated oesophageal reflux. (A) Dark staining lymphocytes within core of papilla, which is in continuity with the lamina propria (H&E). (B) Normal control showing few intraepithelial or subepithelial CD3+ T cells (red staining). (C) Contrasting findings in milk allergic reflux, of dense subepithelial and basal epithelial CD3+ infiltration. (D) CD4+ cells localised to basal epithelium and elongated papillae in another case of milk allergic reflux. The specimen has curled during freezing, leaving surface epithelium on top and at bottom right. (E) Serial section showing CD8+ cells with similar distribution. Smaller numbers of CD25+ cells were seen in identical positions on further serial section. (F) Strong HLA-DR immunoreactivity in basal epithelium and papillae, as well as on submucosal vascular endothelium, in milk allergic reflux. (G,H) Dense eotaxin immunoreactivity within basal (G) and papillary (H) epithelium in two cases of milk allergic reflux (substantially increased compared to controls and primary mechanical reflux).

trafficking. The molecular basis of the observed eotaxin upregulation is unknown, although its antigen specific expression can be induced by T_H2 T cells, and blocked by anti-CD3 monoclonals.[23]

The colocalisation of activated lymphocytes with enhanced eotaxin expression in basal/papillary epithelium is consistent with this, and potentially suggests a mechanism of mucosal homing of lymphocytes activated within the small intestine. Recent murine studies showed a pathway in which enteric antigen induces an eotaxin and IL-5 dependent recruitment of eosinophils and T_H2 T cells in sensitised animals, which disrupt enteric neurones to induce dysmotility.[24] The motility disturbance of CMSE associated reflux may thus occur as a neurological consequence of inflammatory infiltration induced from lamina propria into the epithelial compartment.[24] Intriguing recent evidence suggests that inhaled aeroallergens may also contribute to allergic oesophagitis through a similar eotaxin dependent mechanism.[25] Such mechanisms clearly contrast with the luminally induced inflammation found in primary reflux oesophagitis.

T cell infiltration has previously been reported in reflux oesophagitis.[13–15] We have not yet characterised the T cells for cytokine secretion or expression of the CCR3 chemokine receptor through which eotaxin signalling is transduced. Our finding of upregulated epithelial HLA-DR, however, implies local increased interferon γ production, and thus a mixed $T_H1/$

T_H2 pattern is likely, as IL-5 secretion is also required for mucosal eosinophilia.[17] Similar mixed T_H1/T_H2 responses occur in murine pulmonary eosinophilia.[26]

Despite the immunohistological findings, routine histology was abnormal in only half of these children, although pH testing was abnormal in almost all. In particular, our cases did not show enhanced epithelial eosinophil infiltration, and thus upregulation of eotaxin in cows' milk induced oesophagitis may not reflect as straightforward a role in eosinophil recruitment as proposed for inflammatory bowel disease.[16] This may relate to the requirements for chemokine receptor signalling. Interaction with the chemokine receptor CCR3 is required for effective transduction of eotaxin signalling—this receptor is expressed by several cell types including eosinophils, basophils, and subsets of T_H2 polarised memory T lymphocytes.[27–29] Effective signal transduction through CCR3 may be modulated in several ways, with an obligatory role for IL-5[30] and a facilitatory role of mast cells[31] and CCR3^{+} T cells[27] in eotaxin induced eosinophil recruitment. Mucosal IL-5 is notably increased by antigen challenge in adults with food allergy.[32] By contrast, transforming growth factor β (TGF-β) inhibits the response of CCR3^{+} memory T cells to eotaxin,[29] and it is thus notable that small intestinal lymphocytes in infants with CMSE show low TGF-β expression.[33] Finally, of particular significance in oesophageal reflux, there is evidence of notable pH dependency of CCR3 signalling, with a tenfold decrease in both eotaxin binding and CCR3 mediated signalling at pH 7.0 compared to 7.6.[34] If epithelial permeability is increased within the oesophagus in oesophagitis, it is likely that acid reflux will inhibit eosinophil accumulation, whereas alkaline reflux would promote mucosal eosinophilia, at a given level of eotaxin expression.

In conclusion, we present preliminary evidence to suggest a distinct epithelial chemokine distribution in infantile cows' milk induced oesophageal reflux. This is consistent with recent murine evidence that eotaxin is a critical mediator of experimental allergic dysmotility, through recruitment of eosinophils and T_H2 cells which disrupt enteric neural function.[17 22 24 25] However, further studies on larger numbers of infants are clearly required to determine whether eotaxin is similarly important in human infants in the pathogenesis of allergy associated reflux. If this is confirmed in more extensive studies, eotaxin may become an attractive potential target for specific immunotherapy.

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