

Original article

Spontaneous T_H1 cytokine production by intraepithelial but not circulating T cells in infants with or without food allergies

Background: It has been established that the maintenance of immunological tolerance to dietary antigen and the intestinal flora (oral tolerance) is an actively-maintained process dependent upon mucosal lymphocyte populations. Early life exposures appear critical in the development of such tolerance. However little is known about the activation status of mucosal lymphocytes in human infancy and childhood.

Patients and methods: We have performed flow cytometric analysis for cell lineage and cytokine-production status in peripheral blood and duodenal intraepithelial lymphocytes taken during endoscopy from 20 children [median age 2.9 ± 0.6 years (median $\pm$ SE)] in whom investigation found no intestinal abnormalities (histologically normal controls) and 30 children (median age 1.6 ± 0.4 years) with confirmed allergy to cow's milk and other dietary antigens.

Results: Regardless of clinical status, spontaneous production of cytokines was low or undetectable in peripheral blood cells. By contrast, intraepithelial CD4 and CD8 cells isolated from the small intestine were often activated, with 5% or more showing spontaneous production of T_H1 type [interleukin-2, interferon (IFN)- γ] cytokines in both normal controls and food-allergic children. Stimulation *in vitro* strongly induced cytokine production in peripheral blood but not intraepithelial lymphocytes. Immunohistochemistry showed similar density of IFN- γ^+ intraepithelial lymphocytes in controls and allergic children.

Conclusions: Duodenal intraepithelial lymphocytes in human infants show a state of increased spontaneous activation compared with peripheral blood lymphocytes, and show no significant impairment of T_H1 responses in food allergic children.

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Most antigens encountered by the immune system gain access to the body via the mucosal surfaces. The mucosal immune system is anatomically and functionally distinct from that found elsewhere in the body, and many of the characteristics of systemic immune responses may not apply to those occurring in mucosal tissue (1). In particular, there is evidence that mucosally derived lymphocytes are difficult to activate *in vitro* but may show markers suggestive of *in situ* activation (2, 3). However, little is known about the state of activation of mucosal lymphocytes in the small intestine in early life, in particular whether spontaneous production of cytokines occurs amongst T cell populations. This may be important in the maintenance of unresponsiveness to dietary and luminal antigens within the gut.

Oral tolerance, in which systemic immunological tolerance to a specific protein is induced by orally administering the protein, may be actively mediated by specific lymphocyte populations, which suppress

reactivity of surrounding lymphocytes of variable specificity by a process termed 'bystander tolerance' (4). If this process breaks down, immunologically mediated inflammation occurs (5). Experimental models of inflammatory bowel disease confirm that responses in very early life within the gut-associated lymphoid tissue may determine whether tolerance is generated towards the gut flora, or whether silent sensitization occurs (6). Similar mechanisms maintain tolerance to dietary antigen, and thus the question arises whether what appears to be an increasing incidence of food allergies in developed world children (7, 8) may relate to defective induction of mucosal immune tolerance with changing environmental exposures (9, 10), as suggested for classic atopy in the 'clean child' hypothesis (11). There is evidence that reduced exposure to enteric, rather than airborne, pathogens in childhood may indeed be associated with the development of atopy (10). In this study we examine the characteristics of mucosal lymphocyte subpopulations compared with

peripheral blood lymphocytes in infants both with and without food allergies, as part of an investigation of the establishment of oral tolerance in early life.

Phenotypic and functional investigation of the epithelial layer of the small intestine in humans has in the past been restricted to studies of histological sections. Flow cytometry provides a quantitative and qualitative method of analysing multiple biological parameters of individual cells in a single cell suspension (SCS), without destroying the integrity of the cell architecture. This technology is ideal for analysing lymphocytic subpopulations, and fluorescence activated cell sorting (FACS) analysis using a panel of monoclonal antibodies to cell surface and intracellular molecules, also make it possible to define and study functional properties of lymphoid subpopulations such as activation status and cytokine elaboration (12). Analysis using the ELISPOT technique has suggested spontaneous production of T_H1 and T_H2 cytokines may occur in whole biopsy specimens (13). However the technique requires extended incubation at 37°C and does not allow secure determination of cell lineage. We have thus attempted to determine using flow cytometric analysis whether intraepithelial CD4 and CD8 lymphocytes are spontaneously activated to produce T_H1 cytokines in the small intestine of young children, and whether differences are seen in children who have developed food allergies.

Patients and methods

We performed flow cytometric analysis of spontaneous cytokine production in 50 children, 20 of whom [median age 2.9 ± 0.6 years (median ± SE), range 0.9–10.1] were investigated to exclude gastrointestinal pathology but found to have normal small intestinal biopsies (histologically normal controls) and 30 others (median age 1.6 ± 0.4 years, range 0.3–4.6) with allergy to dietary antigens. These children were endoscoped on clinical grounds because of poor weight gain, chronic diarrhoea or symptoms suggestive of gastro-oesophageal reflux.

Fourteen cases presented with classic immediate reactions to dietary antigen (predominantly egg, milk, peanut, sesame or nuts) (14). These responses, including skin rashes, urticaria, lip swelling or wheezing occurred within 1 h of ingestion. Sixteen cases showed delayed hypersensitive responses (eczema, diarrhoea, vomiting) following milk, egg, soya or wheat ingestion. These reactions usually occurred after 12–24 h following ingestion, and could be confirmed on formal challenge. All showed clear clinical improvement on subsequent exclusion diets, with recurrence of symptoms on open challenge or double-blind placebo-controlled food challenge as clinically indicated. All challenges were supervised by designated medical staff, with nurse specialists and paediatric dieticians. Investigation of the food allergic children found median immunoglobulin A (IgA) 0.5 g/l (upper quartile 0.8), IgE 20 kIU/l (upper quartile 75). Duodenal biopsies showed minor increase in mucosal mononuclear cells or eosinophils in nine of 30, villous blunting in seven of 30 and normal appearances in 14 of 30. Extensive clinical and immunological details of 12 of these patients are reported elsewhere (15).

In addition to mucosal biopsy study, *in vitro* activation of peripheral blood mononuclear cells (PBMC's) was performed using blood obtained from 10 of the histologically normal control children and an

additional nine healthy adult volunteers. These findings were compared with mucosal lymphocyte responses to the same mitogenic stimulus. Further immunohistochemical analysis of interferon (IFN)- γ protein expression were also performed in serial biopsies from 21 of these patients, seven of whom were histologically normal controls, and 14 with food allergy.

Intestinal tissue

Additional biopsy specimens were obtained during routine endoscopy, performed under general anaesthetic, from the fourth part of the duodenum from each patient and were placed in sterile cell dissociation solution (Sigma, Gillingham, UK) and rapidly processed within 15 min. Ethical approval for extra biopsies was obtained from our local Research Ethics Committee and informed consent was obtained in all cases. Single cell suspensions suitable for flow cytometry analysis were prepared following the protocol described by Madrigal et al. (16). First, the epithelial compartment was dissociated from the lamina propria by incubation in calcium-free Hanks' balanced salts solution. The epithelial compartment obtained was agitated continuously for 1 h at 37°C, and the single cell suspension then pelleted from the supernatant and washed once with 0.01M phosphate buffer solution (PBS) supplemented with bovine serum albumin (BSA; 0.2%) and sodium azide (0.02%) (PBS/A/A; Sigma). This compartment thus consisted of a combination of epithelial cells and intraepithelial lymphocytes, and was rapidly obtained without the need for enzymatic tissue digestion. The epithelial cell suspension was resuspended in 1 ml PBS/A/A and was kept at 4°C until incubated with primary fluorochrome-conjugated antibodies for flow cytometric analysis (no longer than 1 h).

Preparation of peripheral blood mononuclear cells

Intracellular cytokine analysis was performed in whole blood cell suspensions as shown by Sewell et al. (17). Peripheral blood from patients and controls (250 μ l) was first added to culture medium (RPMI-1640 [Gibco BRL, Paisley, UK], 500 μ l). To release cytokines from the rough endoplasmic reticulum and maintain them intracellularly, Brefeldin A (GolgiPlug) and Monensin (GolgiStop) were added, according to the manufacturer's recommendation (PharMingen, Oxford, UK), together with ionomycin, which makes the cell permeable to the lectin (Sigma) (16–18). The cells were stimulated with the phorbol myristate acetate (PMA, 'stimulated cultures') or without ('unstimulated cultures') for 4 h at 37°C in a humid, 5% CO₂ atmosphere. After incubation, erythrocytes were lysed with Optilyse C (0.5 ml for 15 min, Coulter, High Wycombe, UK). Cell pellets were resuspended in 1 ml RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (Gibco BRL) and were kept at 4°C until stained.

Multicolour staining of cell preparations

The cells from peripheral blood and the small intestinal epithelial compartment were fixed conventionally with 4% paraformaldehyde at room temperature (RT) for 30 min, and then permeabilized in a 1% saponin solution (Sigma) (PBS/A/A-saponin) for 20 min. One hundred microlitre cell suspensions were dispensed into a 96 wells microwell plates and centrifuged. The supernatants were aspirated and the pellets resuspended in 25 μ l of PBS/A/A-saponin. Typically 10 μ l of each fluorochrome-conjugated monoclonal antibody specific for the surface lineage-marker antigens CD4-ECD and CD8-PC5 (Beckman-Coulter, UK) and the antibodies for cytokine detection [interleukin (IL)-2-FITC and IFN- γ -PE, R & D systems, UK] were added according to the manufacturer's recommendation.

Cells were incubated at room temperature for 30 min in the dark and then washed with PBS/A/A. Finally the cells were fixed by resuspension in 100 μ l of 1% paraformaldehyde-PBS/A/A. Isotype-matched and concentration matched control antibodies, mouse immunoglobulin (Ig)G1-FITC, ECD, PC5 and IgG2a-PE, (Coulter-Immunotech, Bedford, UK) were used to determine nonspecific reactivity and set the optimum fluorescence quadrant markers for the first 18 patients. With relatively small numbers of intestinal lymphocytes for study, we combined this with study of an unstimulated PBL control, which was then used to determine limits for positive cytokine staining in all patients.

Acquisition and analysis of flow cytometric data

The protocol for acquisition of flow cytometric data is shown in Fig. 1. A Coulter Epics-XL-MCL four-colour flow cytometer (Beckman-Coulter, High Wycombe, UK) was used for these experiments. WinList Version 4.0 (Verity) was used to analyse the listmode data. For each analysis, a tight scatter region was drawn around the major lymphocyte population (lymphocyte gate), determined conventionally on the basis of forward and side-scatter of light (representing cell size and granularity respectively), and the events within these regions were acquired using colour compensation. On a lymphocyte-gated plot of CD4 vs CD8, rectangular regions were set to include all the CD4+ bright cells, and all the

CD8+ bright cells. We set a minimum of 1000 events within CD4+ and CD8+ gates for further analysis. Gated one-dimensional histogram plots of the CD4+ cells and CD8+ cells were then generated, with the lower limit for distinguishing cytokine-positive cells being set for each antibody from the matched PBMC unstimulated cell sample and the staining from nonspecific isotype control antibodies. All data are expressed as either the percentage of cytokine-positive CD4+ or CD8+ bright cells.

Immunohistochemical localization of interferon- γ

Paraffin-embedded formalin-fixed tissue sections, taken during the endoscopic procedure, were used in the study. Endogenous peroxidase was blocked with 1% H₂O₂ in tris-buffered saline (TBS). Slides were then pre-incubated with TBS containing 1% saponin (Sigma, Poole, Dorset), and digested with trypsin at 37°C for 5 min. Standard avidin-biotin immunohistochemistry (Vectastain elite®, Vecta, UK) was used for detection of INF- γ immunoreactive cells. Sections were incubated first with blocking serum (horse serum 1:100) for 30 min, then incubated with the primary antibody for 1 h at room temperature (rabbit anti-human IFN- γ 1:450; Serotec, Oxford, UK), before application of the biotinylated secondary antibody and the peroxidase-conjugated avidin complex. Thorough washing with TBS was performed between each step. Bound peroxidase was visualized using 3, 3'-diaminobenzidine, followed by

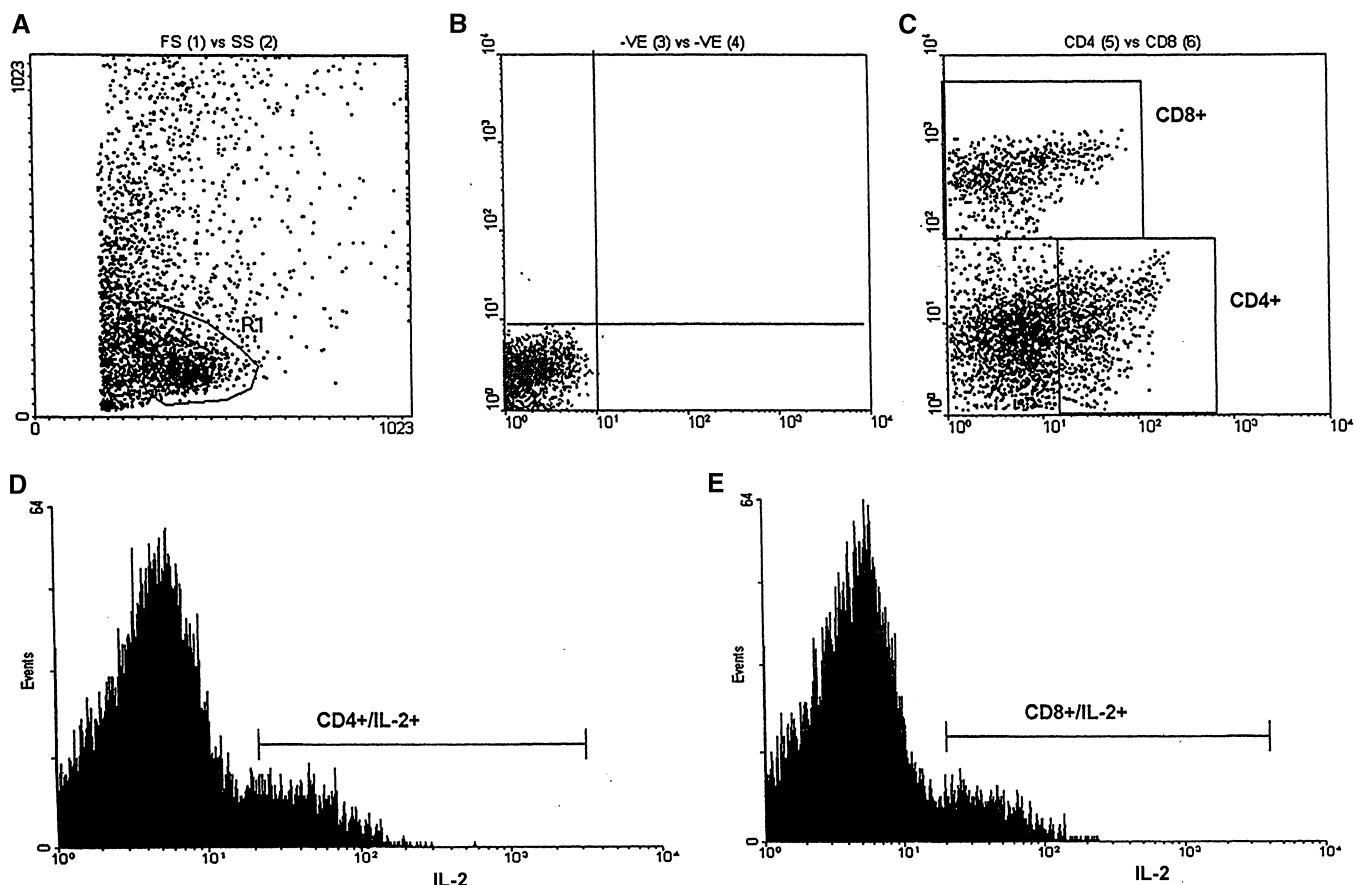


Figure 1. Methodology for flow cytometric analysis. A tight scatter region was drawn around the major lymphocyte population (lymphocyte gate), determined conventionally on the basis of forward and side-scatter of light (A). On a lymphocyte-gated plot of CD4 vs CD8, rectangular regions were set to include all the CD4+ bright cells, and all the CD8+ bright cells (B). Gated one-dimensional histogram plots of the CD4+ cells (C) and CD8+ cells (D) were then generated, allowing detection of cytokine-producing cells.

Table 1. Lymphocyte subsets in peripheral blood and intraepithelial compartment from small bowel biopsy. Result are expressed as the mean percentage $\pm$ SD of the total lymphocytes, within a tight scatter region was draw around the major lymphocyte population (forward scatter/side scatter plot)

Study group	CD4+	CD8+
Inactivated blood	40.6 $\pm$ 4.6	26.3 $\pm$ 3.7
Activated blood	26.2 $\pm$ 10.3*	17.8 $\pm$ 5*
IEL's: normal	11.9 $\pm$ 2.3	25 $\pm$ 3
IEL's: food allergy	11.3 $\pm$ 8.8	19.5 $\pm$ 9.4

* Statistically significant with regard to inactivated blood group, $P < 0.05$.

counterstaining with Mayer's haematoxylin, dehydration and mounting. Staining was first optimized using specimens of tonsil, normal and inflamed colon (Crohn's disease), using negative controls of normal rabbit serum and an irrelevant polyclonal antibody (placental lactogen; Dako, Ely, UK). Ligand inhibition studies using recombinant IFN- γ (Serotec) for overnight preincubation confirmed inhibition of staining. During the staining runs, sections were incubated without primary antibody and with normal rabbit serum as negative controls. Because binding of IFN- γ to matrix glycosaminoglycans was seen within colonic and duodenal biopsies, although

not in tonsil, we characterized the effects of preincubation for 2 h at 37°C with heparanase II 10 μ ml or chondroitinase ABC 1 μ ml (Sigma) prior to staining.

Statistical analysis

Means were compared with two-tailed Student's *t*-test and when data did not reach normality criteria the two-tailed Mann-Whitney *U*-test was used. Values were considered statistically significant if P was < 0.05 .

Results

Lymphocyte subpopulation distribution by flow cytometry

Cell viability amongst intraepithelial lymphocytes was $>95\%$ as assessed by trypan blue staining and flow cytometry using 7-amino actinomycin D (7-AAD; Pharmingen; data not shown). The overall distribution of CD4+ and CD8+ cells in peripheral blood was as previously reported (Table 1) (19). There was marked

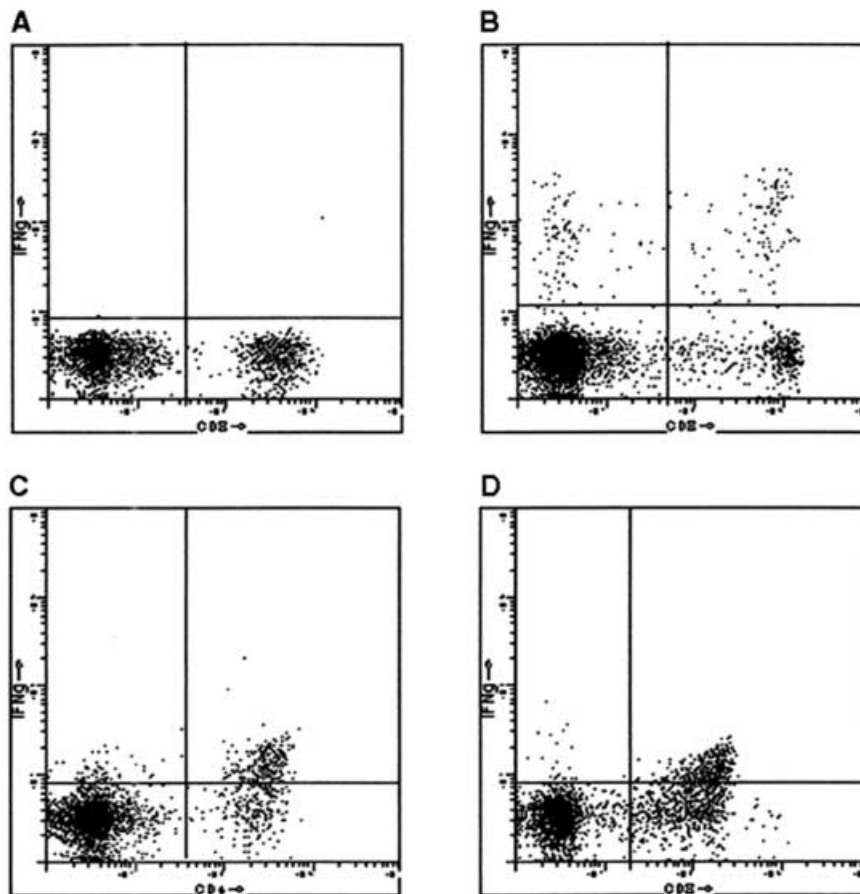


Figure 2. Four quadrant dot plots showing simultaneous flow cytometric analysis for IFN- γ (Y axis) and lineage markers (X axis) in circulating (A and B) and intraepithelial (C, D) cells. The blood had been cultured with (B) or without (A) stimulation by phorbol myristate acetate (PMA) and ionomycin for 4 h before cell harvest. The plots were gated from a tight region around the lymphocyte population defined on forward scatter/side scatter dot plot. A. Intracellular IFN- γ production by CD8+ cells in peripheral blood (upper right quadrant) is almost undetectable in basal conditions (A). (B) IFN- γ induced in peripheral CD8 cells by activation. (C) Spontaneous production in the minority CD4 IEL population. (D) Spontaneous production in CD8 IEL's.

difference in the distribution of CD4+ and CD8+ cells within the small intestinal epithelial compartment compared with peripheral blood, with CD8+ cells dominating over CD4+, as previously recognized (1, 20). In addition, a minority subpopulation of CD4+CD8+ cells (<10%) could be detected, possibly related to extrathymic lymphocyte maturation (20, 21).

Spontaneous T_H1 cytokine production by gut derived lymphocytes

Intracellular cytokine production by CD4+ cells in peripheral blood was almost undetectable in basal conditions, and cytokine-positive cells were rarely seen (<1% in each case, often <0.05%). By contrast the spontaneous production of the T_H1 cytokines IL-2 and IFN- γ was significantly increased amongst CD4+ and CD8+ cells within the lymphocyte region in the small intestinal epithelial compartment compared with nonstimulated PBMC ($P < 0.001$) in both the normal controls and allergic patients (Figs 2 and 3). This was detected in each subject, with no significant differences between the disease groups. The number of CD4/CD8 double positive cells was usually lower than the 1000 limit that we had set for formal analysis, but it was notable that production of cytokines was substantially higher (*c.* 50%) in this subpopulation than in single-positive cells, consistent with a putative role for these cells in immune regulation (22).

Differential effects of *in vitro* activation

We initially studied the effects of *in vitro* activation in both peripheral blood and intestinal lymphocytes. The overall percentage of CD4+ and CD8+ cells in peripheral blood were decreased after the 4 h PMA incubation (Table 1), probably as a consequence of monensin toxicity, as previously reported (20). In contrast to the strong enhancement of cytokine production induced in peripheral blood (Figs 2 and 3), PMA incubation affected intestinal-derived lymphocytes in a different way, in most cases actually reducing cytokine production, as has been reported with a variety of mitogenic stimuli in gut-derived lymphocytes (2, 3). This may reflect either the requirement for CD2-mediated activation or direct cell toxicity (3). In view of these very variable responses, of uncertain physiological relevance, we discontinued *in vitro* stimulation in favour of study of spontaneous activation status.

Immunohistochemical detection of IFN- γ

Within specimens of tonsil or mucosal lymphoid tissue, individual IFN- γ cells could be identified, with little extracellular localization (Fig. 4). By contrast, within both colon and duodenum, IFN- γ was bound to extracellular matrix components, notably the subepithelial basement membrane surface (Fig. 4). We confirmed binding of IFN- γ to heparan sulphate, as previously noted in immunohistochemical studies of mucosal expres-

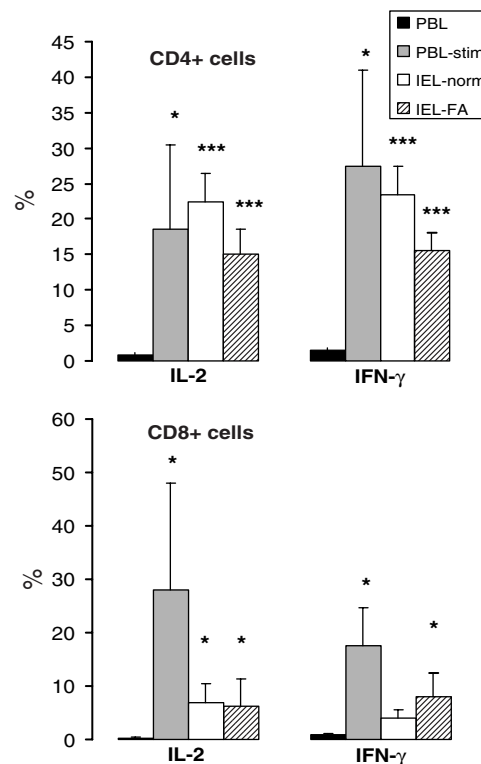


Figure 3. Mean percentage ($\pm$ SE) of IL-2 and IFN- γ cytokine-positive CD4+ (A) and CD8+ cells (B) in PBMC's (inactivated and after 4 h incubation with PMA), compared with IEL's from normal controls and food allergic children. Both groups showed significant increase in IEL production of IL-2 and IFN- γ compared with nonactive PBMC, with no significant differences between groups ($P < 0.001$).

sion (22) or *in vitro* (23, 24) by preincubation with heparanase II, which abrogated this staining, in contrast to chondroitinase ABC, which had no effect.

The study of *in situ* production of IFN- γ in mucosal biopsies showed findings broadly concordant with the flow cytometric data. Immunoreactive IFN- γ + cells could be seen within the epithelial compartment in almost all specimens (Fig. 4), with median density of IFN- γ + intraepithelial lymphocytes (IELs) 2.9 per 100 enterocytes (range 0.9–4.6) in the food allergic children and 2.4 per 100 enterocytes (range 0.4–3.7) in the normal controls (no significant differences between groups detected, $P > 0.3$). Duodenal lymphoid follicles were found within two of the normal biopsies and five from the food allergic children: all showed staining for IFN- γ within individual lymphocytes (Fig. 4). Again, no differences in the proportion of IFN- γ + cells were seen overall between the allergic children and the normal controls.

Discussion

In this study we report the detection by flow cytometric analysis of intracellular cytokines in single cell suspensions of

gut-derived lymphocytes. Flow cytometry has the advantage over other methods of single cell cytokine assessment, such as *in vitro* culture or ELISPOT, of rapid analysis, excluding artefacts of *in vitro* activation or cell death during prolonged culture at 37°C, while allowing secure contemporaneous identification of cell lineage. It is however important to consider potential causes of artefact. There may be contamination of IEL's by lymphocytes derived from the lamina propria. However study of CD19 B cells, which are rare in the epithelial lymphocyte gate but common in the lamina

propria, suggests that this is not a major cause of artefact (25). The presence of dead cells will allow artefactual take-up of fluorescent antibody, thus overestimating cytokine production. Ongoing studies using 7-amino actinomycin D as a marker of cell death show high survival rates in IELs, but not enterocytes (not shown). With these reservations, this technique appears suitable for a wide variety of applications in study of paediatric mucosal disease.

Our results emphasize the differences between lymphocytes located in the intestinal epithelium and those found

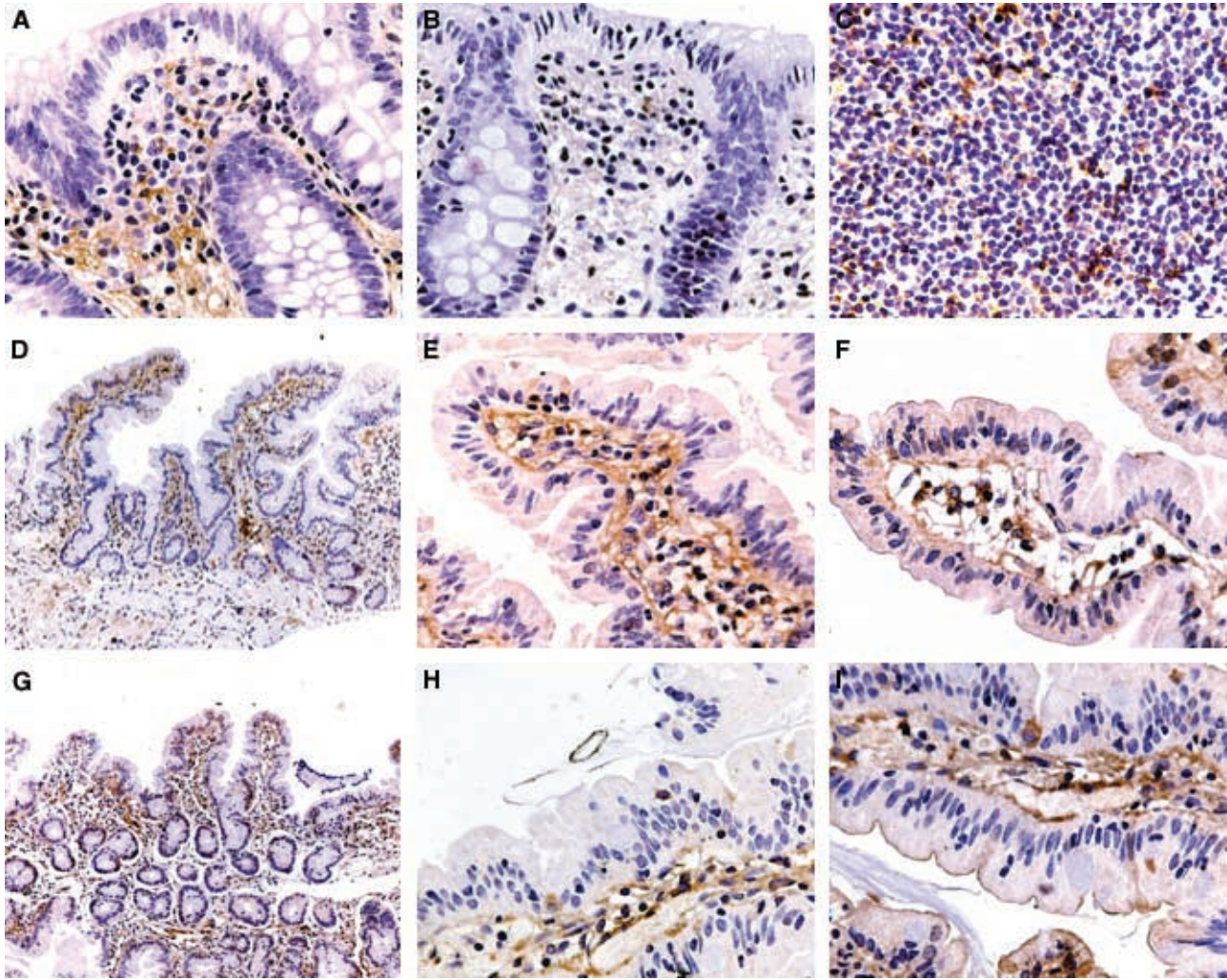


Figure 4. Immunohistochemical localization of IFN- γ , shown as brown staining with counterstaining in blue. A and B represent characterization studies with the IFN- γ polyclonal antibody, C-I findings on representative small intestinal biopsies. Binding of antibody to basement membrane heparan sulphate proteoglycans may be seen in D-I as previously reported (22–24). D-F demonstrate findings in three normal controls, G-I in three children with food allergies. (A) Colonic biopsy from patient with Crohn's disease stained for IFN- γ (original magnification $\times 40$). (B) Serial section stained with IFN- γ antibody following preincubation with recombinant IFN- γ , showing abrogation of staining ($\times 40$). (C) Duodenal lymphoid follicle from child with food allergy, showing individual IFN- γ^+ cells with minimal stromal expression ($\times 40$). (D) Low power view of IFN- γ distribution in normal duodenum, showing predominant expression within the mucosal lamina propria and little expression within submucosa ($\times 10$). (E and F) Individual villi from histologically normal controls showing expression within lamina propria mononuclear cells and some IELs ($\times 40$). (G) Low power view of IFN- γ distribution in mucosa of child with allergy to cow's milk, showing similar expression pattern to normal mucosa shown in D ($\times 10$). (H and I) Individual villi from children with food allergies showing similar findings to controls (E, F) of expression within lamina propria mononuclear cells and some IELs ($\times 40$).

in the circulation. In this study, the spontaneous expression of T_H1 cytokines by IELs but not circulating T cells confirms the importance of local environment in influencing lymphocyte phenotype and thus function. Analysis of the T_H2 cytokine IL-4 was also performed in several children, with no significant differences seen between groups. This forms the basis of ongoing study. It is important to recognize that there is uncertainty about the production of IFN- γ by IELs. Study using PCR technology has suggested that little IFN- γ mRNA may be expressed in IELs in health (26), whereas studies of protein expression using flow cytometry, ELISPOT or immunohistochemistry have suggested that significant numbers of IELs are IFN- γ^+ (27–30). Little is known about the kinetics of IEL trafficking in human infancy, and further studies will be required to elucidate whether mRNA expression occurs prior to homing to the epithelial compartment where protein expression may occur.

Analysis of both cord blood and circulating lymphocytes in allergic children has suggested that IFN- γ generation may be constitutively impaired, leading to T_H2 skewing (31–34). IFN- γ^+ mononuclear cells are reduced in the lamina propria of adult patients with active food allergies (35). However our data support findings by Hauer et al. (13), using ELISPOT, to suggest that generation of T_H1 lymphocytes may be unimpaired in the mucosa of food allergic children. There is evidence that the relative failure to produce IFN- γ by lymphocytes from atopic patients

may reflect low production of T_H1 inducing cytokines such as IL-12 or IL-18 by antigen presenting cells, rather than a primary T cell abnormality (36), and thus our findings may reflect local production of these cytokines within the epithelial compartment. Expression of IL-18 (IFN- γ inducing factor) has indeed been detected within the cytoplasm of murine enterocytes in both foetal and adult life, to an extent higher than in any other tissue (37). However there is as yet little data in human infancy.

These findings are thus consistent with the hypothesis of a continuous physiological inflammation in the small intestine, presumably in response to dietary and bacterial antigens. Several animal models confirm the importance of a balanced mucosal immune response to this ongoing challenge if immunopathology is to be avoided (2, 5, 7). Further study of the pathogenesis of allergic diseases should ideally include mucosally derived, as well as circulating lymphocytes.

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References

- BRANDTZAEG P, BAEKKEVOLD ES, FARSTAD IN, JAHNSEN FL, JOHANSEN FE, NILSEN EM et al. Regional specialization in the mucosal immune system: what happens in the microcompartments? *Immunol Today* 1999;**20**:141–151.
- CHRIST AD, COLGAN SP, BALK SP, BLUMBERG RS. Human intestinal epithelial cell lines produce factor(s) that inhibit CD3-mediated T-lymphocyte proliferation. *Immunol Letters* 1997;**58**:159–165.
- TARGAN SR, DEEM RL, LIU M, WANG S, NEL A. Definition of a lamina propria T cell responsive state. Enhanced cytokine responsiveness of T cells stimulated through the CD2 pathway. *J Immunol* 1995;**154**:664–675.
- GROUX H, POWRIE F. Regulatory T cells and inflammatory bowel disease. *Immunol Today* 1999;**20**:442–446.
- MACDERMONT RP. Alterations of the mucosal immune system in inflammatory bowel disease. *J Gastroenterol* 1996;**31**:907–916.
- MIZOGUCHI A, MIZOGUCHI E, CHIBA C, BHAN AK. Role of appendix in the development of inflammatory bowel disease in TCR- α mutant mice. *J Exp Med* 1996;**184**:707–715.
- GRUNDY J, MATTHEWS S, BATEMAN B, DEAN T, ARSHAD SH. Rising prevalence of allergy to peanut in children: data from 2 sequential cohorts. *J Allergy Clin Immunol* 2002;**110**:784–789.
- HILL DJ, HOSKING CS, HEINE RG. Clinical spectrum of food allergy in children in Australia and South-East Asia: identification and targets for treatment. *Ann Med* 1999;**31**:272–281.
- PÉREZ-MACHADO MA, THOMSON MA, LATCHAM F, ASHWOOD P, SIM R, WALKER-SMITH JA et al. Reduction of TGF- β producing regulatory lymphocytes in the intestinal mucosa of children with multiple food allergy. *Eur J Immunol* 2003;**33**:2307–2315.
- MURCH SH. The immunologic basis for intestinal food allergy. *Curr Opin Gastroenterol* 2000;**16**:552–557.
- YAZDANBAKHSH M, KREMSNER PG, VAN REE R. Allergy, Parasites, and the Hygiene Hypothesis. *Science* 2002;**296**:490–494.
- CARTER LL, SWAIN SL. Single cell analyses of cytokine production. *Curr Opin Immunol* 1997;**9**:177–182.
- HAUER AC, BREESE EJ, WALKER-SMITH JA and MACDONALD TT. The frequency of cells secreting interferon- γ and interleukin -4, -5, and -10 in the blood and duodenal mucosa of children with cow's milk hypersensitivity. *Pediatr Res* 1997;**42**:629–629.
- JOHANSSON SG, HOURIHANE JO, BOUSQUET J et al. A revised nomenclature for allergy. An EAACI position statement from the EAACI nomenclature task force. *Allergy* 2001;**56**:815–824.
- LATCHAM F, MERINO F, WINTER C, LANG A, GARVEY J, THOMSON MA et al. A consistent pattern of minor immunodeficiency and subtle enteropathy in children with multiple food allergy. *J Pediatr* 2003;**143**:39–47.

16. MADRIGAL L, LYNCH S, FEIGHERY C, WEIR D, KELLEHER D, O'FARRELLY C. Flow cytometric analysis of surface major histocompatibility complex class II expression on human epithelial cells prepared from small intestinal biopsies. *J Immunol Methods* 1993;**158**:207–214.
17. SEWELL WA, NORTH ME, WEBSTER AD, FARRANT J. Determination of intracellular cytokines by flow-cytometry following whole-blood culture. *J Immunol Methods* 1997;**209**:67–74.
18. NORTH M.E, IVORY K, FUNACHI M, WEBSTER ADB, LANE AC, FARRANT J. Intracellular cytokine production by human CD4⁺ and CD8⁺ T cells from normal and immunodeficient donors using directly conjugated anti-cytokine antibodies and three-colour flow cytometry. *Clin Exp Immunol* 1996;**105**:517–522.
19. COMANS-BITTER WM, DE GROOT R, VAN DEN BEERND R, NEIJENS HJ. Immunophenotyping of blood lymphocytes in childhood. *J Paediatr* 1997;**130**:388–393.
20. LYNCH S, KELLEHER D, FEIGHERY C, WEIR DG, O'FARRELLY C. Flow cytometric analysis of intraepithelial lymphocytes from human small intestinal biopsies reveals populations of CD4⁺CD8⁺ and CD8 $\alpha\alpha$ ⁺ cells. *Eur J Gastroenterol Hepato* 1993;**5**:907–912.
21. HOWIE D, SPENCER J, DeLORD D, PITZALIS C, WATHEN NC, DOGAN A et al. Extrathymic T cell differentiation in the human intestine early in life. *J Immunol* 1998;**161**:5862–5872.
22. RAQIB R, LJUNGDAHL A, LINDBERG AA, ANDERSSON U, ANDERSSON J. Local entrapment of interferon gamma in the recovery from *Shigella dysenteriae* type 1 infection. *Gut* 1996;**38**:328–336.
23. FERNANDEZ-BOTRAN R, YAN J, JUSTUS DE. Binding of interferon gamma by glycosaminoglycans: a strategy for localization and/or inhibition of its activity. *Cytokine* 1999;**11**:313–325.
24. SADIR R, FOREST E, LORTAT-JACOB H. The heparan sulfate binding sequence of interferon-gamma increased the on rate of the interferon-gamma-interferon-gamma receptor complex formation. *J Biol Chem* 1998;**273**:10919–10925.
25. ASHWOOD P, MURCH SH, ANTHONY A, PELLICER AA, TORRENTE F, THOMSON M et al. Intestinal lymphocyte populations in children with developmental regression and autism: evidence for extensive mucosal immunopathology. *J Clin Immunol* 2003;**23**:504–517.
26. FORSBERG G, HERNELL O, MELGAR S, ISRAELSSON A, HAMMARSTROM S, HAMMARSTROM ML. Paradoxical coexpression of proinflammatory and down-regulatory cytokines in intestinal T cells in childhood celiac disease. *Gastroenterology* 2002;**123**:667–678.
27. CAROL M, LAMBRECHTS A, VAN GOSSUM A, LIBIN M, GOLDMAN M, MASCART-LEMONE F. Spontaneous secretion of interferon gamma and interleukin 4 by human intraepithelial and lamina propria gut lymphocytes. *Gut* 1998;**42**:643–649.
28. VAN DAMME N, DE KEYSER F, DEMETER P, BAETEN D, MIELANTS H, VERBRUGGEN G et al. The proportion of Th1 cells, which prevail in gut mucosa, is decreased in inflammatory bowel syndrome. *Clin Exp Immunol* 2001;**125**:383–390.
29. AL-DAWOUD A, NAKSHABENDI I, FOULIS A, MOWAT AM. Immunohistochemical analysis of mucosal gamma-interferon production in coeliac disease. *Gut* 1992;**33**:1482–1486.
30. OLAUSSEN RW, JOHANSEN FE, LUNDIN KE, JAHNSEN J, BRANDTZAEG P, FARSTAD IN. Interferon- γ -secreting T cells localize to the epithelium in coeliac disease. *Scand J Immunol* 2002;**56**:652–664.
31. WARNER JA, MILES EA, JONES AC et al. Is deficiency of interferon gamma production by allergen-triggered cord blood cells a predictor of atopic eczema. *Clin Exp Allergy* 1994;**24**:423–430.
32. TANG MLK, KEMP AS, THORBURN J, HILL DJ. Reduced interferon- γ secretion in neonates and subsequent atopy. *Lancet* 1994;**344**:983–985.
33. PRESCOTT SL, MACAUBAS C, SMALLACOMBE T, HOLT BJ, SLY PD, HOLT PG. Development of allergen-specific T-cell memory in atopic and normal children. *Lancet* 1999;**353**:196–200.
34. SUOMALAINEN H, SOPPI E, LAINE S, ISOLAURI E. Immunologic disturbance in cows milk allergy. II. Evidence for defective interferon-gamma production. *Pediatr Allergy Immunol* 1993;**4**:203–207.
35. LIN XP, MAGNUSSON J, AHLSTEDT S, DAHLMAN-HOGLUND A, HANSON LLA, MAGNUSSON O et al. Local allergic reaction in food-hypersensitive adults despite a lack of systemic food-specific IgE. *J Allergy Clin Immunol* 2002;**109**:879–887.
36. JUNG T, MOESSNER R, DIECKHOFF K, HEIDRICH S, NEUMANN C. Mechanisms of deficient interferon- γ production in atopic diseases. *Clin Exp Allergy* 1999;**29**:912–919.
37. TAKEUCHI M, NISHIZAKI Y, SANO O, OHTA T, IKEDA M, KURIMOTO M. Immunohistochemical and immuno-electron-microscopic detection of interferon- γ -inducing factor (“interleukin-18”) in mouse intestinal epithelial cells. *Cell Tissue Res* 1997;**289**:499–503.