

Cow's milk protein-specific T-helper type I/II cytokine responses in infants with necrotizing enterocolitis

Chuang S-L, Hayes PJ, Ogundipe E, Haddad M, MacDonald TT, Fell JM. Cow's milk protein-specific T-helper type I/II cytokine responses in infants with necrotizing enterocolitis.

Pediatr Allergy Immunol 2008.

© 2008 The Authors

Journal compilation © 2008 Blackwell Munksgaard

Enteral feeding, in particular with formula feeds, is associated with necrotizing enterocolitis (NEC). In this study, we have examined, in the systemic and mucosal immune compartments, for evidence of bovine milk antigen sensitization in infants with NEC. Eleven newborns with Bell's staging 2–3 NEC [median post-conceptional age 31 wk (range 27–41 wk)], 21 neonatal controls [33 (28–40) wk] and 15 infants undergoing intestinal resection or mucosal biopsy for non-inflammatory conditions [39 (34–42) wk] were studied. Spontaneous and antigen or mitogen elicited interferon- γ (IFN- γ) [T-helper type I (Th1)], interleukin (IL)-4 and IL-5 [T-helper type II (Th2)] responses were enumerated using single-cell enzyme-linked immunospot (ELISPOT) assay in peripheral blood (PBMC) or lamina propria mononuclear cells. NEC infants, compared with controls, showed a significant elevation in baseline PBMC cytokine secreting cells, vigorous mitogen responses (20- to 120-fold increase) for IFN- γ , IL-4 and IL-5 ($p < 0.001$), strong responses to beta-lactoglobulin (β lg) (IFN- $\gamma > \text{IL-4/IL-5}$, $p \leq 0.001$), and somewhat smaller casein responses. Similarly, in the lamina propria, a small but significant increase in spontaneous cytokine-secreting cells was detected in NEC infants ($p < 0.01$), with an IFN- γ /IL-4 predominant phytohemagglutinin (PHA)/concanavalin-A (ConA) response. Three of nine NEC infants (but no controls) also showed a positive ELISPOT response to β lg (IFN- γ only) but none to casein. We have thus demonstrated significant cow's milk protein (CMP) sensitization in NEC, at least in the systemic compartment (mixed Th1/Th2), with minimal mucosal activation in some cases. These novel findings provide a potential mechanism for a direct contributory role of CMP in the pathogenesis of NEC.

**S.-L. Chuang¹, P. J. Hayes²,
E. Ogundipe¹, M. Haddad³, T. T.
MacDonald⁴ and J. M. Fell³**

¹Department of Neonatology, Chelsea and Westminster Hospital, ²Division of Investigative Sciences, Imperial College, ³Department of Paediatric Gastroenterology and Paediatric Surgery, Chelsea and Westminster Hospital, ⁴Centre For Infectious Diseases, Institute of Cell and Molecular Science, Barts and The London, Queen Mary's School of Medicine and Dentistry, London, UK

Key words: newborns; necrotizing enterocolitis; beta-lactoglobulin; casein; interferon-gamma; interleukin-4; Th1/Th2

Dr Shu-Ling Chuang, Consultant Neonatologist, Department of Neonatology, Chelsea and Westminster Hospital, 369 Fulham Road, London SW10 9NH, UK

Tel.: +44 208 8467928

Fax: +44 208 8467792

E-mail: shu-ling.chuang@chelwest.nhs.uk

Accepted 25 January 2008

The pathogenesis of neonatal necrotizing enterocolitis (NEC) is not fully understood. Recognized predisposing factors are pre-maturity, formula feeding, bacterial translocation and intestinal hypoxia/ischaemia (1, 2). Once initiated, a cascade of immunological events ensues, with the release of a myriad of pro- and counter-inflammatory mediators (3, 4), which are postulated to lead to mucosal injury (5, 6).

Neonatal NEC typically occurs in enterally fed infants (1). Epidemiological data have indicated an increased risk of NEC in formula-fed infants compared with infants given expressed breast milk and/or donor breast milk (7). To date, the potential link between formula milk and human NEC has largely been investigated indirectly through study of the immunoprotection conferred by breast milk (8) or differences in the

stool flora and hence gut microbiota in infants fed different milks (9). No specific pathogen has consistently been implicated in NEC (10).

Dietary (milk) antigens, together with microbes, make up the bulk of the novel antigens encountered in the newborn period. The interaction between luminal antigens, at the intestinal mucosal interface, with the vast array of immune effector cells housed within the mucosa and associated lymphoid system, is thought to be critical in the establishment of oral tolerance and systemic immune modulation (11). Preterm infants, with their increased mucosal permeability (12, 13), may be particularly vulnerable to the harmful effects of excess antigen uptake across the mucosal barrier, yet the direct role of cow's milk protein (CMP) on the mucosal and systemic inflammatory cascade in NEC has not been delineated.

It is believed that the T-helper type I/II (Th1/Th2) cytokine balance plays a pivotal role in gut immunoregulation, with a propensity towards Th1 polarization in most human intestinal inflammatory conditions (14, 15). It is therefore conceivable that CMPs may also play a role in the inflammatory cascade in NEC by eliciting harmful Th1 or Th2 responses.

In this study, we have examined the T-cell responses to bovine milk protein antigens in babies with NEC. We characterized and compared the Th1/Th2 cytokine profile in infants who develop NEC compared with normal neonates, with respect to the spontaneous production and the effector responses to *in vitro* stimulation with mitogens or CMPs, in the systemic and mucosal compartments. Non-overlapping signature cytokines of Th1 [interferon- γ (IFN- γ)] and Th2 [interleukin (IL)-4, IL-5] were analysed at a single-cell level using a sensitive enzyme-linked immunospot (ELISPOT) assay (16, 17).

Methods

Patients and samples

The study was conducted at Chelsea and Westminster Hospital, London, UK over an 18-month period (July 2002 – Dec 2003). Eleven consecutive infants with confirmed NEC i.e. grade 2 or 3 on modified Bell's staging (20), were recruited (median post-conceptual age 31/40, range 27–41 wk). Non-inflamed controls were also recruited. These were of term or preterm birth gestation, without suspected NEC or clinical sepsis at the point of tissue or blood collection, (median post-conceptual age 35/40, range 28–42 wk) (Table 1).

Table 1. Baseline characteristics of the infants in the three study groups – NEC, preterm controls (blood samples) and non-inflamed gut resection/biopsy controls; 'Gut controls'

	NEC (n = 11)	Preterm blood controls (n = 21)	'Gut controls' (n = 15)
Birth gestation (wk)	26 (24–30)	27 (23–32)	38 (36–39)
Birthweight (g)	1000 (538–1568)	928 (590–1780)	2500 (1530–3900)
Post-menstrual age (wk)†	31 (27–41)	33 (28–40)	39 (34–42)
Post-natal age (days)*	34 (7–83)	35 (10–96)	3 (3–17)
Gender (male), n (%)	5 (45)	12 (57)	10 (67)
Mode of delivery (LSCS), n (%)	5 (45)	11 (52)	6 (40)
Initiation of enteral feeding, n (%)	10 (91)	21 (100)	11 (73)
Exclusive breast milk, n (%)†	8 (80)	16 (76)	9 (82)

Values are medians (ranges).

*Denotes the time of tissue collection.

†Expressed as a percentage of the infants in the group in whom enteral feeds were initiated.

LSCS, caesarian section; NEC, necrotizing enterocolitis.

The spectrum of disease severity of the 11 infants with NEC by modified Bell's staging was as follows: stage 2b (n = 2); stage 3a (n = 3); stage 3b (n = 6). Nine of 11 infants underwent laparotomy. Concurrent blood culture-positive septicaemia was present in three infants (coagulase-negative staphylococcus, *Candida albicans*, and mixed *Klebsiella oxytoca* and coagulase-negative staphylococcus). Five NEC infants developed intestinal perforations. Six infants died, one from massive haemolysis and disseminated intravascular coagulation, four in the immediate post-operative period and one from late chronic lung disease. Blood for cytokine analysis was collected from all subjects at the acute phase of NEC (immediately prior to surgery if undergoing laparotomy), and taken at the same time as routine blood testing or from an indwelling arterial line. Primary ileal involvement was found in all nine NEC subjects who underwent laparotomy. Mucosa was harvested from intestinal resection margins. Histological confirmation of NEC was shown in all cases.

Blood specimens were obtained from 21 healthy preterm infants matched for birth gestation, birth weight and post-menstrual age to the NEC infants. Intestinal control specimens were obtained from 15 neonates ('gut controls') undergoing intestinal resections or biopsies for the following clinical indications: diagnostic rectal mucosal biopsies for delayed passage of meconium (n = 7; six normal, one with non-inflamed aganglionosis), colonic mucosa from colostomy

formations for anorectal anomalies ($n = 3$) or long segment Hirschsprungs ($n = 1$) and duodenal/jejunal mucosa of small bowel atresias ($n = 4$). The control mucosal specimens were histologically normal. Peripheral blood was obtained from 14 of the 15 patients for comparative cytokine analysis.

This study received research ethics approval from the Riverside Ethics Committee. Informed parental consent was obtained.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were separated from 0.5 ml of heparinized blood by density gradient centrifugation using standard procedures. Cells were washed twice in complete medium (R10), containing RPMI-1640 (Sigma-Aldrich Co., Poole, UK), 50 U/ml penicillin, 50 µg/ml streptomycin, 10 µg/ml gentamicin, 2 mM L-glutamine, sodium pyruvate, 10 mM hepes buffer and supplemented with 10% heat-inactivated foetal calf serum (FCS). Concentration of the recovered PBMC, resuspended in 2 ml of medium, was determined using a Z1 particle counter (Beckman Coulter, High Wycombe, UK). Percentage viabilities were determined using trypan blue exclusion. Cell suspensions were diluted in R10 to a concentration of 1×10^6 viable cells/ml (or 0.5×10^6 for NEC blood).

Isolation of lamina propria mononuclear cells

Biopsies/surgical mucosal specimens were washed extensively in complete medium and lamina propria mononuclear cells (LPMCs) were obtained by collagenase digestion using a previously described protocol with minor modifications (18). Briefly, the epithelium was first removed by incubation in 20 ml of Ca/Mg free Hanks balanced solution (HBBS, Sigma) containing 1% EDTA for 45 min at room temperature. Mucosal fragments were then transferred to a RPMI medium (with 10% FCS) containing 1.5 mg/ml type IV collagenase (Sigma) and incubated at 37°C, with vigorous pipetting at 10- to 15-min intervals. Complete enzymatic digestion was achieved in 2–3 h, with mucosal fragments becoming macroscopically invisible. Cells were filtered through a 100 µm nylon cell strainer (BD Falcon, Oxford, UK), washed three times in RPMI medium and resuspended in 1 ml of R10 medium (or 0.5 ml for biopsy specimens). Cell numbers, viability (>95% in all) and purity (<5% enterocyte contamination) were determined as above.

Detection and enumeration of IFN- γ , IL-4 and IL-5 cytokine-secreting cells

ELISPOT assays were performed as described elsewhere (19, 20). Capture and detection monoclonal antibodies (mAb) were purchased from Mabtech AB, Nacka Strand, Sweden. Nitrocellulose 96-well plates (MAIPS 4510; Millipore, Watford, UK) were precoated with cytokine-specific capture mAb (10 µg/ml) in 100 µl of phosphate buffer saline (PBS), washed and blocked 2 h with R10 medium (200 µl/well) at 37°C in a 5% CO₂ humidified atmosphere. Freshly isolated PBMC or LPMC, in 100 µl of R10 medium, were seeded per well, in triplicate and duplicate, respectively. Cells were incubated for 20–24 h, in the absence or presence of respective antigens or mitogens: phytohaemagglutinin (PHA) (10 µg/ml), concanavalin-A (ConA) (10 µg/ml), beta-lactoglobulin (βlg) (500 µg/ml) and casein (500 µg/ml). The optimal antigen concentrations and duration of incubation were determined in preliminary experiments. At the end of incubation, the wells were washed seven times with PBS/0.05% Tween 20 (Sigma). A biotinylated cytokine-specific mAb (1 µg/ml, 100 µl/well in PBS/0.5% BSA) was added and left at room temperature for 3–4 h. After six washes with PBS/Tween, avidin–biotin–peroxidase complex, prepared per manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA), was added for an hour at room temperature. The wells were washed three times with PBS/Tween, followed by three times with PBS. Finally, 100 µl of amino-ethyl-carbazole substrate was added per well and incubated at room temperature for 4 min. Spot development was terminated by gently running under tap water. The wells were dried over night in the dark prior to image analysis.

Evaluation of spots

The number of spots formed per well was enumerated using computer assisted automated ELISPOT Reader System and KS ELISPOT software (Carl Zeiss, Welwyn Garden City, UK). The final results were expressed as spot-forming cells per 10^5 input mononuclear cells (SFC/ 10^5 MNC), averaged for each sample and antigen based on the replicate samples.

Statistical analysis

Data were presented as medians [(interquartile range (IQR)]. In each group, the data points were tested for normality of distribution. For non-parametric data distribution, Mann–Whitney

U-test (two-tailed) was used for the calculation of differences in the SFC between different patient groups and Wilcoxon signed rank test for the comparison of values before and after antigen stimulation. Statistical significance was assumed for differences with a *p*-value of ≤ 0.05 .

Results

ELISPOTs were performed for three cytokines, IFN- γ and IL-4 and IL-5 on PBMCs from the three groups (11 NEC, 21 preterm neonatal controls and 14/15 'gut controls'). There were insufficient cells for IL-5 analysis in one NEC patient. Results are expressed as number of spot-forming cells per 10^5 mononuclear cells (SFC/ 10^5 MNC). Where *in vitro* stimulation was performed, antigen-specific response are represented by Δ ELISPOTs i.e. the number of spot-forming cells post-antigen stimulation minus the number of spontaneous unstimulated spot-forming cells (SFC/ 10^5 MNC).

The PBMC ELISPOT response of infants with NEC compared with preterm controls

Spontaneous ELISPOTs. In normal preterm controls, the frequencies of cells spontaneously secreting cytokines IFN- γ , IL-4 and IL-5 in the blood were almost undetectable (median < 1 SFC/ 10^5 cells). In contrast, a higher number of IFN- γ , IL-4 and IL-5 secreting cells could be detected in the blood of NEC patients ($p < 0.001$ for IFN- γ , $p < 0.001$ for IL-4 and $p < 0.001$ for IL-5), and in similar frequencies for all three cytokines (Table 2).

Mitogen stimulation. *In vitro* stimulation of PBMC with PHA and ConA produced a very small increase in the number of IFN- γ , IL-4 and IL-5 secreting cells in the controls, but a vigorous response in the NEC subjects. The number of cytokine secreting cells detectable in the NEC group outnumbered that in the controls by 20- to 120-fold, for all three cytokines ($p < 0.001$) (Table 2).

Antigen-specific responses to β -lactoglobulin. β -lactoglobulin stimulation elicited negligible or no responses in the majority of normal controls [median increase (Δ ELISPOTs) IQR for IFN- γ , 0 (0, 1); IL-4, 1 (0, 2); IL-5, 0 (-1, 2)] (Fig. 1). However, in NEC infants, high numbers of β lg-specific cytokine secreting cells were detected using PBMCs (at least 70- to 120-fold that seen in the controls). The strongest response was for IFN- γ secreting cells, followed

Table 2. Peripheral blood spontaneous and mitogen-induced cytokine responses in NEC, preterm controls and 'Gut controls' (ELISPOT)

ELISPOTS (SFC/ 10^5 MNC)					
	NEC (n = 11)	Preterm controls (n = 21)	p-value	'Gut controls' (n = 14)	p-value
Spontaneous					
IFN- γ	19 (9–53)	0 (0–2)	0.00024	0 (0–2)	0.00068
IL-4	17 (9–59)	0 (0–2)	0.00042	0 (0–2)	0.00087
IL-5	23 (4–69)	1 (0–3)	0.00053	1 (0–4)	0.0031
PHA					
IFN- γ	412 (285–717)	7 (3–47)	0.000010	18 (2–48)	0.000031
IL-4	484 (237–748)	4 (1–14)	0.000007	6 (1–21)	0.000031
IL-5	353 (189–463)	8 (1–14)	0.000013	3 (0–12)	0.000007
ConA					
IFN- γ	285 (210–402)	15 (2–89)	0.000048	10 (6–146)	0.00020
IL-4	288 (210–461)	11 (1–24)	0.000007	5 (2–31)	0.000085
IL-5	363 (207–527)	6 (1–17)	0.000020	8 (1–39)	0.000046

Values are spot-forming cells in median and interquartile ranges. *p*-value refers to comparison of cytokine secreting cell frequency, spontaneous and post-mitogen stimulation, between NEC and control groups (Mann–Whitney *U*, two-tailed test). There were insufficient cells for IL-5 analysis in one NEC patient.

by IL-4 and IL-5 (Δ ELISPOTs for IFN- γ , 71 (49, 244); IL-4, 49 (33, 190); IL-5, 57 (22, 232); $p < 0.001$).

Antigen-specific responses to casein. Casein stimulation of PBMCs from the controls and NEC patients produced a similar pattern of response to that induced by β lg (Fig. 2). Minimal responses were elicited in the controls for all three cytokines. There was no response in the controls for IFN- γ , one control had minimal IL-4 response and two controls had minimal IL-5 responses that were ≥ 10 SFC/ 10^5 MNC above the baseline. In contrast, in the NEC group, a significant rise in IFN- γ secreting cells was produced in the peripheral blood following casein stimulation in the majority of infants. This casein-specific-IFN- γ response was, however, much less than that induced by β lg [median increase (Δ ELISPOT) by 12 vs. 71 SFC/ 10^5 MNC for casein and β lg, respectively]. The increases in IL-4 and IL-5 secreting cells post-casein treatment were not significantly different between the NEC and control subjects.

The mucosal ELISPOT response of infants with NEC compared with controls

It was not possible to obtain age-matched control gut mucosal samples for infants without NEC, so we compared the responses of children with NEC to tissues obtained from infants undergoing surgical resection or mucosal biopsy at term.

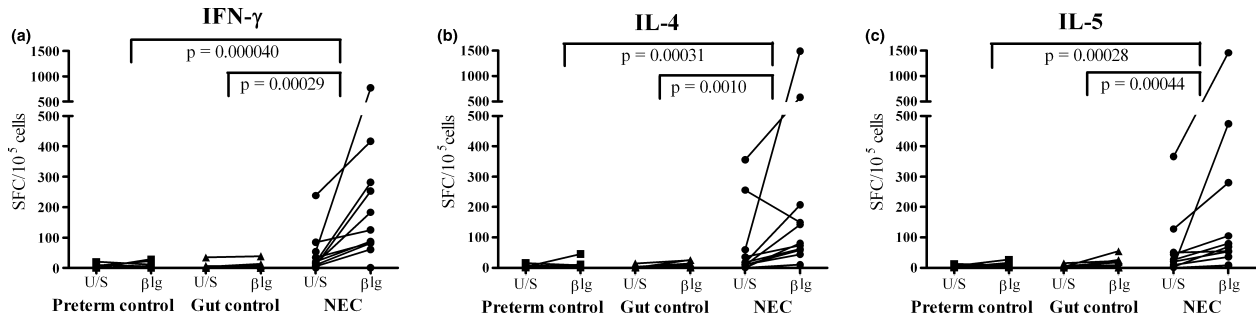


Fig. 1. Peripheral blood mononuclear cell ELISPOT responses to beta-lactoglobulin (β lg). Freshly isolated peripheral blood mononuclear cells from necrotizing enterocolitis (NEC) infants, matched preterm controls and ‘Gut controls’ were incubated with or without β lg for 20 h. The frequency of IFN- γ (a), IL-4 (b), IL-5 (c) secreting cells is expressed as spot-forming cells per 10^5 mononuclear cells (SFC/ 10^5 MNC). Each line represents a single patient before and after β lg stimulation. p-value refers to the comparison of β lg-specific cytokine responses i.e. number of SFC elicited in the presence of β lg minus number of SFC in the absence of β lg, in NEC infants vs. controls (Mann–Whitney U , two-tailed test). U/S denotes unstimulated.

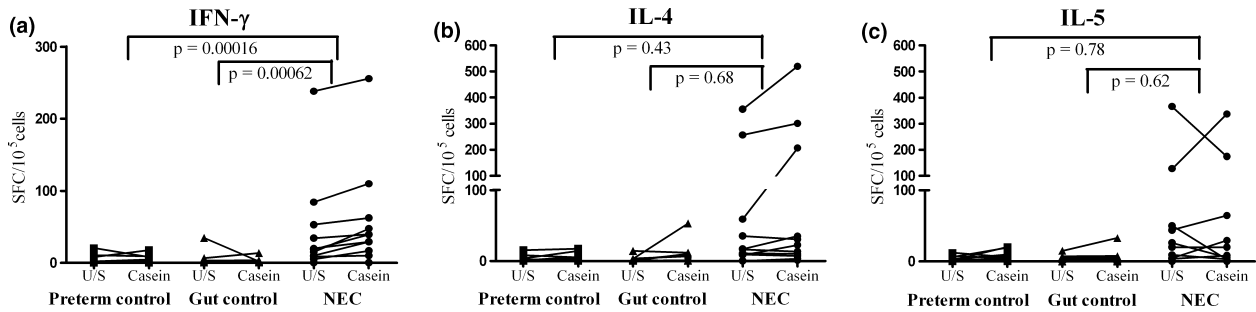


Fig. 2. Peripheral blood mononuclear cell ELISPOT responses to casein. Freshly isolated peripheral blood mononuclear cells from necrotizing enterocolitis (NEC) infants, matched preterm controls and ‘Gut controls’ were incubated with or without casein for 20 h. The frequency of IFN- γ (a), IL-4 (b), IL-5 (c) secreting cells generated before and after casein stimulation, is shown here for each patient, expressed as spot-forming cells per 10^5 mononuclear cells (SFC/ 10^5 MNC). p-value refers to the comparison of casein-specific responses i.e. number of casein elicited ELISPOTs minus spontaneous cytokine production, in NEC infants vs. controls (Mann–Whitney U , two-tailed test). U/S denotes unstimulated.

Spontaneous ELISPOTs. LPMC from controls showed no spontaneous cytokine secreting cells. NEC infants, on the other hand, demonstrated a small number of spontaneous IFN- γ and IL-4 cytokine secreting cells which was significantly greater than the controls ($p \leq 0.005$). IL-5 secretion was not significantly different between NEC and control infants (Table 3).

Mitogen stimulation. PHA stimulation elicited negligible or no lamina propria IFN- γ , IL-4 or IL-5 cytokine response in the controls. In NEC patients, the median frequency of IFN- γ and IL-4 spot-forming cells following PHA stimulation was three- to fourfold that of unstimulated baseline values. ConA stimulation produced similar results to PHA, with absence of response in the majority of controls and an increase, mainly, in the number of IFN- γ and IL-4 cytokine secreting cells in NEC infants. A very small increase in IL-5 spot-forming cells was seen in both the controls and NEC infants with

Table 3. Lamina propria mononuclear spontaneous and mitogen-induced cytokine responses in infants with NEC and ‘Gut controls’ (ELISPOT)

	ELISPOTS (SFC/ 10^5 MNC)		
	NEC (n = 9)	‘Gut controls’ (n = 15)	p-value
Spontaneous			
IFN- γ	3 (0–15)	0 (0–0)	0.0031
IL-4	2 (1–9)	0 (0–1)	0.0015
IL-5	1 (0–4)	0 (0–1)	0.13
PHA			
IFN- γ	11 (5–41)	1 (0–2)	0.0019
IL-4	6 (0–31)	0 (0–1)	0.019
IL-5	3 (1–4)	0 (1–4)	0.18
ConA			
IFN- γ	16 (10–38)	1 (0–7)	0.0011
IL-4	13 (4–20)	1 (0–2)	0.00037
IL-5	6 (3–14)	3 (1–6)	0.087

Values are spot-forming cells in median (interquartile ranges). p-values refer to comparison of cytokine secreting cell frequency, spontaneous and post-mitogen stimulation, between NEC and control groups (Mann–Whitney U , two-tailed test). There were insufficient cells for IL-5 analysis in one NEC patient and one gut control.

mitogen stimulation but this was not significantly different between groups (Table 3).

Antigen-specific responses of LPMCs from infants with NEC and controls. None of the control samples showed a lamina propria ELISPOT response to β lg or casein, for all three cytokines. A third of NEC subjects, on the other hand, showed an increase in IFN- γ positive cells following β lg incubation by >5 SFC/ 10^5 MNC above the baseline (unstimulated) value. The maximal rise above the baseline was only 13 SFC/ 10^5 MNC. Casein induced IFN- γ ELISPOTs were not detected in NEC subjects. No distinct pattern emerged for IL-4 or IL-5 mucosal responses with dietary antigenic challenge.

Finally, as these mucosal ELISPOT comparisons were of necessity done with a more mature gestational age control population than the PBMC experiments, we repeated the ELISPOT assays with blood samples from these term 'Gut control' infants for further comparison with results obtained from the preterm control infants. As with blood samples from preterm infants that had been age-matched for the NEC cases, PBMC ELISPOTs were low in the predominant term neonatal 'gut controls'. No significant differences were observed when compared with preterm blood controls for the three studied cytokines (Table 2, Figs 1 and 2).

Discussion

We present here, for the first time, clear evidence of significant CMP sensitization in human neonatal NEC, primarily in the systemic compartment, with relatively minor mucosal activation. The bovine milk protein directed effector response is of both Th1 and Th2 type at the systemic level. This contrasted markedly with the findings in normal neonatal controls, where both the PBMC and mucosal lamina propria cells remained quiescent under identical stimulatory conditions.

The concomitant Th1 and Th2 systemic cytokine up-regulation observed in almost all NEC patients following *in vitro* CMP challenge (to both β lg and casein) is clearly of interest in light of the purported importance of formula-based milk feeds in the aetiology of NEC and difficulties with feed re-establishment in some infants following NEC. The response was greatest in IFN- γ positive cells although significant increases in IL-4 and IL-5 secreting cells were also found. In contrast, systemic CMP reactivity was demonstrated by only a very small proportion of preterm controls, and notably at low

frequencies. This finding is similar to a previous report that showed absence of cord blood recall antigen response to CMP in preterm infants under 34-wk gestation (19).

In the intestine, *in vitro* cytokine response to β lg was elicited in the lamina propria cells in just three of nine NEC neonates but none in the controls. This was IFN- γ predominant as opposed to the mixed Th1/Th2 pattern in the blood. Casein stimulation failed to elicit significant response in either NEC or control subjects. The mucosal cytokine response in NEC infants was considerably lower than that of blood with regards to both spontaneous production and following antigen stimulation. These mucosal specimens were, however, harvested from the resection margins in NEC subjects. Thus although they provide a useful insight into the state of immune activation of the tissues left *in situ*, they might under-represent of the inflammatory changes upstream at the site of maximal insult and necrosis. Furthermore, the experimental mucosal responses may well have been further dampened by several potential mechanisms such as the absence of co-stimulatory agents, the presence of local immunoregulatory factors, or a decrease in T cells and/or antigen presenting cell numbers because of chemotaxis or apoptosis.

There are no previous human data on neonatal mucosal milk antigen responses in infants with NEC. The *in vivo* effects of formula milk on intestinal cytokine expression in NEC have so far been investigated only in animal experimental models. A trend towards an up-regulation in IFN- γ was observed in a formula milk induced neonatal rat NEC model (20), whilst in the acidified intraluminal casein neonatal NEC piglet model, significant attenuation in T-cell density was observed in both the affected and proximal viable zone (21). Our findings are consistent with these observations.

Animal models have traditionally employed casein. The role of β lg in NEC has not been previously evaluated. In our study, β -lactoglobulin appears to be the more immunogenic of the two CMPs studied. The concentrations of milk antigens used in this study were the same as those used by others (16, 22). Factors governing allergenicity of bovine milk proteins and mechanistic involvements in mucosal inflammation or of food allergy are complex (23) and as yet unclarified in many aspects.

In this pilot study, NEC subjects with concurrent sepsis were not excluded as bacterial translocation from breached mucosa barrier or perforation may occur as part of the disease process. However, of the three NEC subjects

with documented positive blood cultures, one had a Gram-negative sepsis and was the only infant with NEC who failed to show a cytokine response to *in vitro* β lg stimulation. A full dietary history prior to the onset of disease was not always attainable particularly in the NEC subjects born in another hospital. This limited the analysis and interpretation of milk antigen response according to milk feed history.

The preterm blood controls in our study were closely matched to NEC neonates. It is clear that spontaneously activated IFN- γ , IL-4 or IL-5 secreting cells are barely detectable in the PBMC from healthy preterms. The controls showed virtually no cytokine secreting cells at rest, and only weak mitogen response across all three studied cytokines, albeit with a slight excess of IFN- γ .

It is also notable that significant basal activation of Th2 in parallel with Th1 cytokine secreting cells in approximately the same orders of magnitude has been demonstrated here in NEC infants for the first time. The pattern was further amplified 20- to 120-fold with mitogen stimulation. To our knowledge, there have been only two other studies evaluating systemic Th2 cytokine contribution in NEC, reporting either undetectable (i.e. normal) spontaneous IFN- γ and IL-4 levels by ELISA method or a low and essentially unaffected IL-4 or IL-5 (one-tenth of IFN- γ) levels in the blood of NEC infants using flow cytometry (24, 25). This discrepancy with our observations probably reflects methodological differences. The ELISPOT technique used here, in addition to its validated sensitivity, has the advantage of enabling comparison to be made of the cell numbers secreting different cytokines, in lieu of absolute values (15). Our results thus add to the body of evidence that preterm newborns have the ability to up-regulate inflammatory responses under appropriate conditions, although in the case of NEC, in a dysregulated and potentially destructive fashion.

The availability of uninflamed neonatal control mucosal specimens is inevitably constrained by the clinical characteristics of the patient groups who require biopsy or resection. This explains the 'gut controls' in our study being predominant from term neonates with obstructive pathologies and their relative earlier age of presentation. We found, however, no significant difference in the blood cytokine profiles between the two control cohorts (in 'gut controls' and preterm controls) and in their respective comparisons with the NEC population. Similarly, in lamina propria cells of normal 'gut controls', an absence of constitutive cytokine secretion and

response to mitogen stimulation was observed. In contrast, a small but significant rise in the frequency of spontaneous cytokine secreting cells was detected in the mucosa specimens of NEC infants (IFN- γ > IL-4 > IL-5), with *in vitro* up-regulation in mainly IFN- γ and IL-4 positive cells (IFN- γ > IL-4) in response to PHA or ConA stimulation. Previous reports on mucosal IFN- γ transcripts in human NEC have been conflicting (26, 27), whilst the IL-4 or IL-5 cytokines, to our knowledge, have not been evaluated in normal neonatal intestinal mucosa or in human NEC. In our study, spontaneous IFN- γ , IL-4 or IL-5 cytokine secreting cells were not detected in the control mucosa of either ileal/jejunal (n = 4) or colonic/rectal (n = 11) origin. This difference in mucosal cytokine responses observed between control and NEC neonates thus could not be attributed to the preponderance of small bowel mucosa in the latter.

The profound *in vitro* systemic cytokine response to bovine milk proteins demonstrated by NEC infants in this study could be indicative of a primary patho-physiological event but could equally well represent a secondary response to milk antigens arriving in the systemic compartment via an altered gut barrier. A substantial number of the NEC neonates in our study had received exclusive breast feeding. It has, however, been demonstrated previously in other studies that CMP exposure could occur via the transplacental route (28, 29), in breast milk (30) or from the formula-based fortifiers commonly used in the extremely premature or growth restricted infant. In addition, the use of formula feeds has been associated with a transient increase in intestinal permeability in human infants and animals (12).

As already mentioned, cell numbers were particularly low in the mucosa of neonatal controls. Validation of antigen-specific responses against a negative antigen control using an irrelevant antigen could therefore not be performed. It is probable that non CMP-dietary antigens encountered by the infants, for example, through breast milk may also be involved. Further work using dietary antigens other than cow's milk would help elucidate if the response is only specific to CMP.

With the increased mucosal permeability of the immature gut or in the severely compromised gut mucosal barrier in NEC, one could speculate that milk antigens may freely escape into the systemic circulation. Whether the release of Th1 and Th2 mediators from the activated immune cells with CMP challenge, as shown in this study, has any role in exacerbating local mucosal or systemic inflammation at this point is conjectural.

Conclusion

The cause of NEC is likely to be multifactorial, a consequence of synergy of several potential risk factors. It is unlikely that any of the major implicated factors is the sole mechanism responsible for the disease development. The findings from this study provide a potential mechanism by which CMP may be involved in the pathogenesis of NEC by directly contributing towards and potentially exacerbating the inflammatory cascade. Further studies are required to elucidate the clinical relevance of these *in vitro* findings, which could well be particularly important, for example, in defining the optimal feed regimen for infants recovering from NEC.

References

- STOLL BJ. Epidemiology of necrotizing enterocolitis. *Clin Perinatol* 1994; 21: 205–18.
- NEU J. Necrotizing enterocolitis: the search for a unifying pathogenic theory leading to prevention. *Pediatr Clin North Am* 1996; 43: 409–32.
- EDELSON MB, BAGWELL CE, ROZYCKI HJ. Circulating pro- and counterinflammatory cytokine levels and severity in necrotizing enterocolitis. *Pediatrics* 1999; 103: 766–71.
- VISCARDI RM, LYON NH, SUN CC, HEBEL JR, HASDAY JD. Inflammatory cytokine mRNAs in surgical specimens of necrotizing enterocolitis and normal newborn intestine. *Pediatr Pathol Lab Med* 1997; 17: 547–59.
- CLAUD EC, WALKER WA. Hypothesis: inappropriate colonization of the premature intestine can cause neonatal necrotizing enterocolitis. *FASEB J* 2001; 15: 1398–403.
- HSUEH W, CAPLAN MS, TAN X, MACKENDRICK W, GONZALEZ-CRUSSI F. Necrotizing enterocolitis of the newborn: pathogenetic concepts in perspective. *Pediatr Dev Pathol* 1998; 1: 2–16.
- LUCAS A, COLE T. Breast milk and neonatal necrotising enterocolitis. *Lancet* 1990; 336: 1519–23.
- GOLDMAN AS. Modulation of the gastrointestinal tract of infants by human milk. Interfaces and interactions. An evolutionary perspective. *J Nutr* 2000; 130: 426S–31S.
- YOSHIOKA H, ISEKI K, FUJITA K. Development and differences of intestinal flora in the neonatal period in breast-fed and bottle-fed infants. *Pediatrics* 1983; 72: 317–21.
- PETER CS, FEUERHAHN M, BOHNHORST B, et al. Necrotising enterocolitis: is there a relationship to specific pathogens? *Eur J Pediatr* 1999; 158: 67–70.
- KELLY D, COUTTS AG. Early nutrition and the development of immune function in the neonate. *Proc Nutr Soc* 2000; 59: 177–85.
- INSOFT RM, SANDERSON IR, WALKER WA. Development of immune function in the intestine and its role in neonatal diseases. *Pediatr Clin North Am* 1996; 43: 551–71.
- ROBERTSON DM, PAGANELLI R, DINWIDDIE R. Milk antigen absorption in the preterm and term neonate. *Arch Dis Child* 1982; 57: 141–5.
- MONTELEONE I, VAVASSORI P, BIANCONI L, MONTELEONE G, PALLONE F. Immunoregulation in the gut: success and failures in human disease. *Gut* 2002; 50: III60–4.
- HANSSON T, DANNAEUS A, KLARESKOG L. Cytokine-producing cells in peripheral blood of children with coeliac disease secrete cytokines with a type 1 profile. *Clin Exp Immunol* 1999; 116: 246–50.
- HAUER AC, BREESE EJ, WALKER-SMITH JA, MACDONALD TT. The frequency of cells secreting interferon-gamma and interleukin-4, -5, and -10 in the blood and duodenal mucosa of children with cow's milk hypersensitivity. *Pediatr Res* 1997; 42: 629–38.
- HELMS T, BOEHM BO, ASAAD RJ, TREZZA RP, LEHMANN PV, TARY-LEHMANN M. Direct visualization of cytokine-producing recall antigen-specific CD4 memory T cells in healthy individuals and HIV patients. *J Immunol* 2000; 164: 3723–32.
- BELL MJ, TERNBERG JL, FEIGIN RD, et al. Neonatal necrotizing enterocolitis. Therapeutic decisions based upon clinical staging. *Ann Surg* 1978; 187: 1–7.
- HAUER AC, RIEDERER M, GRIESSL A, ROSEGGER H, MACDONALD TT. Cytokine production by cord blood mononuclear cells stimulated with cows milk proteins *in vitro*: interleukin-4 and transforming growth factor beta-secreting cells detected in the CD45RO T cell population in children of atopic mothers. *Clin Exp Allergy* 2003; 33: 615–23.
- NADLER E, DICKINSON E, KNISELY A, et al. Expression of inducible nitric oxide synthase and interleukin-12 in experimental necrotizing enterocolitis. *J Surg Res* 2000; 92: 71–7.
- ANTTILA A. T-cell-mediated mucosal immunity is attenuated in experimental necrotizing enterocolitis. *Pediatr Surg Int* 2003; 19: 326–30.
- NAGATA S, MCKENZIE C, PENDER SLF, et al. Human Peyer's patch T Cells are sensitized to dietary antigen and display a Th cell type 1 cytokine profile. *J Immunol* 2000; 165: 5315–21.
- HEINE R, ELSAYED S, HOSKING C, HILL DJ. Cow's milk allergy in infancy. *Curr Opin Allergy Clin Immunol* 2002; 2: 217–25.
- JURGES ES, HENDERSON DC. Inflammatory and immunological markers in preterm infants: correlation with disease. *Clin Exp Immunol* 1996; 105: 551–5.
- NG PC, LI K, WONG RP, et al. Proinflammatory and anti-inflammatory cytokine responses in preterm infants with systemic infections. *Arch Dis Child Fetal Neonatal Ed* 2003; 88: F209–13.
- PENDER SL, BRAEGGER C, GUNTHER U, et al. Matrix metalloproteinases in necrotising enterocolitis. *Pediatr Res* 2003; 54: 160–4.
- FORD H, WATKINS S, REBLOCK K, ROWE M. The role of inflammatory cytokines and nitric oxide in the pathogenesis of necrotizing enterocolitis. *J Pediatr Surg* 1997; 32: 275–82.
- SZEPFALUSI Z, LOIBICHLER C, PICHLER J, REISENBERGER K, EBNER C, URBANEK R. Direct evidence for trans-placental allergen transfer. *Pediatr Res* 2000; 48: 404–7.
- JONES AC, MILES EA, WARNER JO, COLWELL BM, BRYANT TN, WARNER JA. Fetal peripheral blood mononuclear cell proliferative responses to mitogenic and allergenic stimuli during gestation. *Pediatr Allergy Immunol* 1996; 7: 109–16.
- KILSHAW PJ, CANT AJ. The passage of maternal dietary proteins into human breast milk. *Int Arch Allergy Appl Immunol* 1984; 75: 8–15.