

# Endotoxin, cytokines and lipid peroxides in children with intussusception

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**Background:** Intussusception is a relatively common paediatric surgical emergency. The aim of this study was to investigate selected inflammatory mediators in children with acute intussusception and to identify potentially useful plasma markers of clinical outcome.

**Methods:** Clinical, radiographic, operative and pathological details were recorded prospectively of all children presenting to a single institution with a confirmed diagnosis of acute intussusception during 1 year. Paired acute and convalescent venous blood samples were collected in a standard manner for blinded analysis of the following: malondialdehyde, C-reactive protein (CRP), interleukin (IL) 6, neopterin, tumour necrosis factor  $\alpha$ , endotoxin, and immunoglobulin (Ig) G and IgM antiendotoxin core antibody (EndoCAb).

**Results:** Thirty-two consecutive children (23 boys, nine girls) with a median age of 4 months were studied. Acute ileocolic intussusception was managed by air enema reduction ( $n = 19$ ), operative reduction ( $n = 8$ ) or surgical resection ( $n = 5$ ). Peripheral blood cultures were sterile. Acute levels of plasma IL-6, neopterin and CRP were significantly raised in comparison to both normal laboratory ranges and convalescent samples ( $P < 0.001$ ). Using stepwise discriminant analysis, CRP was identified as the best variable at distinguishing between the three treatment groups ( $P < 0.001$ ). IgM EndoCAb concentrations were significantly greater in the convalescent sera of all the patients ( $P < 0.001$ ).

**Conclusion:** Acute ileocolic intussusception in childhood is associated with endotoxaemia and significantly raised levels of circulating inflammatory cytokines. Plasma CRP at diagnosis showed a statistically significant positive correlation with disease severity.

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## Introduction

Intussusception is one of the commonest abdominal emergencies in childhood. The incidence of the condition in Western societies is approximately 2.3 per 1000 live births and almost two-thirds of cases occur in infancy<sup>1,2</sup>. Untreated acute ileocolic intussusception usually progresses to intestinal strangulation, perforation, sepsis and fatal multiple organ failure. Even with appropriate treatment, some children with apparently uncomplicated intussusception become severely ill and develop multiple

organ failure<sup>3</sup>. The key inflammatory mediators involved in acute intussusception have not been studied systematically. In this study, plasma cytokines (interleukin (IL) 6 and tumour necrosis factor (TNF)  $\alpha$ ), free radicals (lipid peroxides) and endotoxin were investigated in children with intussusception, with the aim of achieving a better understanding of the disease process and in an attempt to identify potentially useful markers of disease outcome.

## Patients and methods

The parents of all children presenting to a single institution during 1 year (1997–1998) with a confirmed diagnosis of intussusception were invited to participate in the study;

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written informed parental consent was obtained in each case. Clinical, radiographic, operative and pathological details were recorded for each patient. In addition to routine haematological investigations, a further 5 ml of peripheral venous blood was collected in a standard manner for subsequent analysis ('acute sample'). A second blood sample was obtained from each child after full recovery before discharge home and processed in an identical manner ('convalescent sample'). Stool, midstream urine and peripheral blood cultures were also obtained from each patient.

For the endotoxin assays, free-flowing blood samples were collected into tubes without additive using a no-touch technique, centrifuged at 626*g* for 10 min, and the serum stored at  $-75^{\circ}\text{C}$ . For cytokine, malondialdehyde (MDA) and C-reactive protein (CRP) estimations, free-flowing venous blood was collected into ethylenediamine tetraacetic acid Vacutainer<sup>TM</sup> tubes (Becton, Dickinson and Company, Plymouth, UK), centrifuged at 626*g* for 10 min, and the plasma was stored in aliquots at  $-75^{\circ}\text{C}$ . Haemolysed samples were discarded.

Assays were performed for MDA, CRP, IL-6, neopterin, TNF- $\alpha$ , endotoxin, immunoglobulin (Ig) G antiendotoxin core antibody (IgG EndoCAB) and IgM antiendotoxin core antibody (IgM EndoCAB) by independent investigators blinded to the clinical details of individual patients. Each laboratory was subject to strict quality control and all assays were undertaken using validated methods. Interassay and intra-assay coefficients of variation were below 10 per cent.

This study was carried out after obtaining institutional ethics committee approval and in accordance with institutional ethical guidelines.

### Cytokine assays

Plasma samples were assayed for circulating TNF- $\alpha$ , IL-6 and neopterin in duplicate using commercially available enzyme-linked immunosorbent assays (EASIA assays; Medgenix Diagnostics, Fleurus, Belgium).

### Malondialdehyde assay

MDA, a marker of lipid peroxidation, was assayed by high-performance liquid chromatography (HPLC) as described previously<sup>4</sup>. Boiling serum in acid solution containing thiobarbituric acid achieved simultaneous hydrolysis of lipid peroxide and reaction of the generated MDA. Excess production of MDA from non-lipid peroxide sources was inhibited by the addition of butylated hydroxytoluene. The reaction was stopped by immersing the tubes in iced water. The coloured product was then extracted with butanol/ethyl acetate, evaporated to dryness, redissolved in the HPLC mobile phase and injected on to the HPLC column,

where it was separated by reverse-phase HPLC and measured at 532 nm.

### C-reactive protein assay

Plasma levels of CRP were assayed in duplicate by an immunoturbidimetric method using commercially available kits according to the manufacturer's recommendations (Tina-quant CRP; Roche, Welwyn Garden City, UK). The normal plasma CRP concentration in children is below 5 mg/l.

### Endotoxin assay

Endotoxin was measured by the Chromogenix colorimetric kinetic limulus amoebocyte lysate kit method (Chromogenix, New York, USA). Samples were diluted 1 : 10 in pyrogen-free water and heat treated at  $85^{\circ}\text{C}$  for 10 min. The kit endotoxin standard was diluted in 1 : 10 normal serum in pyrogen-free water (heat treated) and made up in a series of 12 doubling dilutions from 24 endotoxin units (EU)/ml for the quantitative standard curve. Time to onset of the reaction was measured at 405 nm optical density in a Thermomax reader (Molecular Devices, Woking, UK) and results determined in EU from the standard curve using SoftMax software (Molecular Devices)<sup>5</sup>.

### Endogenous antiendotoxin core antibody assays

EndoCABs were measured by methods described previously<sup>5,6</sup>. In brief, microplates were coated with equimolar amounts of polymyxin complexes of an Rc or Rd lipopolysaccharide from each of four Gram-negative species and blocked with bovine serum albumin. Dilutions of test sera (1 : 200) were added in triplicate, and eight doubling dilutions of a calibrated pooled serum standard with a high IgG EndoCAB titre were run on each plate as a quantitative standard curve. After incubation and washing, plates were developed with  $\gamma$ -chain-specific (IgG) or  $\mu$ -chain specific (IgM) alkaline phosphatase conjugates (Zymed, San Francisco, California, USA), followed by para-nitro phenylphosphate alkaline phosphatase substrate (Sigma-Fast; Sigma, Poole, UK). Optical densities at 405 nm were read on an automated plate reader (Molecular Devices) with SoftMax software. Results were determined in median units (100 median units = median of healthy adult IgG or IgM EndoCAB normal range).

### Statistical analysis

Statistical comparisons between acute and convalescent values were made by using the Wilcoxon signed rank test for

paired non-parametric data. Comparison between subgroups (non-operative; surgery, no resection; surgery and resection) was by the Kruskal–Wallis test. The data were normalized by natural logarithmic transformation primarily to enable discriminant analysis, which was used to determine which variable was best at distinguishing between the three subgroups. Statistical significance was accepted at  $P < 0.05$ . SPSS for Windows (Version 8; SPSS, Chicago, Illinois, USA) was used for the analysis.

## Results

### Clinical features

Of 35 consecutive children with acute intussusception, 32 (23 boys and nine girls) with a median age of 4 (range 2–19) months were recruited. Parents of two children declined to enrol their child in the study, and one child with a postoperative small bowel intussusception was not included. Symptoms and signs of intussusception were present for a median of 2 (range 1–8) days before referral and most commonly comprised abdominal pain ( $n = 30$ ), bilious vomiting ( $n = 26$ ), bloody stools ( $n = 24$ ) and a palpable abdominal mass ( $n = 20$ ). Duration of symptoms was not significantly longer in infants who needed intestinal resection. Three children had experienced a recent symptomatic upper respiratory tract infection or gastroenteritis.

After initial assessment (including blood tests) and resuscitation with 4.5 per cent human albumin solution (median volume 20 (range 10–140) ml/kg), an ileocolic intussusception was confirmed in all children by abdominal ultrasonography. In each case, prophylactic intravenous broad-spectrum antibiotics and morphine analgesia were

administered followed by an attempt at air enema reduction. A successful non-operative reduction was achieved in 19 children (19 of 27 reducible intussusceptions) using a median sustained pressure of 100 (range 60–120) mmHg. Thirteen children required laparotomy; five needed intestinal resection for an irreducible intussusceptum. No intussusception had reduced spontaneously before laparotomy. Histological examination of resected specimens confirmed the presence of non-viable bowel with lymphoid hyperplasia in four and a gangrenous Meckel's diverticulum in one patient. There were no deaths. All recovered uneventfully, except one girl who required four laparotomies, an extensive small bowel resection, parenteral nutrition and a temporary ileostomy. She subsequently made a full recovery.

### Laboratory data

All peripheral blood cultures were sterile but two children had a concomitant urinary tract infection (enterococcus, coliform) and two had pathogens isolated from stool cultures (*Campylobacter* sp. and rotavirus). Routine haematological and biochemical results were within normal limits on admission, except for mild hypoalbuminaemia in six patients and a raised peripheral white blood cell count in three children.

Acute levels of plasma IL-6 and neopterin were significantly raised in comparison to both normal laboratory ranges and levels in convalescent samples (Table 1). TNF- $\alpha$  concentrations were raised in both acute and convalescent samples but there was no significant overall difference between these two samples. Statistically significant differences between acute samples in the operative and

**Table 1** Plasma values in 32 children with acute ileocolic intussusception

	Non-operative ( $n = 19$ )	Operative reduction ( $n = 8$ )	Intestinal resection ( $n = 5$ )	All children ( $n = 32$ )
IL-6 (pg/ml)				
Acute	16.9 (10.2–29.9)	24.0 (14.4–37.6)	17.2 (9.3–42.4)	18.8 (10.6–30.4)
Convalescent	2.0 (2.0–5.7)	9.3 (3.2–58.3)	3.5 (2.4–281)	3.0 (2.0–9.2)*
MDA ( $\mu\text{mol/l}$ )				
Acute	2.66 (2.26–3.00)	2.77 (2.44–3.10)	1.84 (1.78–2.32)	2.59 (2.18–2.92)
Convalescent	2.80 (2.54–3.32)	3.07 (2.37–3.58)	2.50 (2.20–3.12)	2.86 (2.39–3.33)
TNF- $\alpha$ (pg/ml)				
Acute	28.1 (19.6–36.6)	44.1 (30.7–64.7)	30.9 (29.6–63.7)	30.6 (24.7–38.5)
Convalescent	37.3 (16.3–47.6)	57.4 (30.6–64.8)	32.8 (25.2–47.1)	39.8 (20.5–50.5)
Neopterin (ng/ml)				
Acute	1.62 (1.17–1.82)	2.82 (2.49–3.16)	2.74 (2.07–3.13)	1.88 (1.62–2.73)
Convalescent	1.09 (0.39–1.50)	1.42 (1.06–2.16)	1.53 (1.42–1.83)	1.20 (1.04–1.68)*

Values are median (interquartile range). IL, interleukin; MDA, malondialdehyde; TNF, tissue necrosis factor. Normal adult reference ranges: IL-6  $< 8.5$  pg/ml; MDA 2.4–6.1  $\mu\text{mol/l}$ ; TNF- $\alpha$   $< 20$  pg/ml; neopterin  $< 2.48$  ng/ml. \* $P < 0.001$  versus acute (Wilcoxon signed rank test)

**Table 2** Plasma C-reactive protein concentrations in 32 children with acute ileocolic intussusception

	Plasma C-reactive protein (mg/l)			
	Non-operative reduction ( <i>n</i> = 19)	Operative reduction ( <i>n</i> = 8)	Intestinal resection ( <i>n</i> = 5)	All children ( <i>n</i> = 32)
Acute	36.1 (28.9–38.4)	54.6 (48.3–57.9)	129.7 (119.4–136.2)	38.9 (35.2–57.0)
Convalescent	8.3 (3.9–9.0)*	9.1 (5.4–9.9)*	15.2 (10.7–17.0)*	8.8 (4.8–9.7)*

Values are median (interquartile range). \**P* < 0.001 *versus* acute (Wilcoxon signed rank test). *P* < 0.001 between subgroups for acute samples (Kruskal–Wallis test)

**Table 3** Endotoxin and antiendotoxin core antibody levels in acute and convalescent samples from 32 children with acute intussusception

	Non-operative reduction ( <i>n</i> = 19)	Operative reduction ( <i>n</i> = 8)	Intestinal resection ( <i>n</i> = 5)	All children ( <i>n</i> = 32)
Endotoxin (EU/ml)				
Acute	0.01 (0–0.03)	0.02 (0.01–0.08)	0.03 (0.02–0.44)	0.01 (0.01–0.04)
Convalescent	0.01 (0–0.10)	0.01 (0–0.11)	0.04 (0–0.22)	0.01 (0–0.09)
IgM EndoCAb (MMU/ml)				
Acute	37.0 (15.6–73.6)	39.2 (26.2–73.5)	31.6 (27.7–195.7)	37.6 (24.8–74.6)
Convalescent	178.1 (78.8–327.3)	157.5 (22.2–205.8)	289.2 (149.9–300.8)	180.4 (63.4–290.9)*
IgG EndoCAb (GMU/ml)				
Acute	38.6 (19.8–60.6)	19.0 (10.3–66.3)	50.0 (21.0–79.1)	31.4 (15.9–56.3)
Convalescent	26.7 (22.6–64.8)	22.3 (5.9–47.8)	61.8 (47.2–84.1)	32.6 (22.2–62.1)

Values are median (interquartile range). Ig, immunoglobulin; EndoCAb, antiendotoxin core antibody. \**P* < 0.001 *versus* acute (Wilcoxon signed rank test)

non-operative groups were observed for neopterin (*P* = 0.001) (Table 1). MDA concentrations were not significantly different in acute and convalescent samples and in patients undergoing intestinal resection. Plasma CRP concentrations were significantly raised in acute samples, both in comparison to levels in convalescent samples and to the normal laboratory reference range (less than 5 mg/l) (Table 2). Statistically significant differences between the acute samples were observed for plasma CRP on subgroup analysis; the CRP concentration was greatest in patients who needed intestinal resection followed by that in patients who had a laparotomy for reduction of intussusception, and least in children who had successful enema reduction (Table 2).

Considering all patients with intussusception, endotoxin concentrations were not significantly different between acute and convalescent samples, taken 2–6 days later in most cases (Table 3). However, levels of IgM EndoCAb were significantly greater in the convalescent sera of all subgroups of patients with intussusception (*P* < 0.001).

Statistically significant differences between acute sample concentrations for the three intussusception subgroups were found for only two variables, CRP and neopterin.

Using stepwise discriminant analysis, CRP was identified as the best variable at distinguishing between the three subgroups (*P* < 0.001), with no further advantage gained from including other variables.

## Discussion

The mortality rate associated with intussusception has declined steadily in developed countries and is now less than 1 per cent<sup>3</sup>. However, serious morbidity is not uncommon, particularly in patients with a delayed diagnosis<sup>2</sup>. The biological mechanisms involved in complicated intussusception are poorly understood, although intestinal ischaemia–reperfusion injury may be important in some cases. The role of free radicals, inflammatory cytokines and endotoxin has been speculative. This study was designed to explore which of these factors might be involved in the systemic aspects of the disease. This knowledge may help to provide an early indicator of disease severity and yield new insights into therapeutic strategies in severe cases.

Hydrostatic or air enema reduction is usually only possible if the intestine is viable and it is almost always unsuccessful in the presence of strangulated intestine.

Consequently, failure of enema reduction is strongly correlated with the more severe forms of intussusception. Overzealous attempts to reduce the intussusception in these severe cases is hazardous. Plasma markers might be valuable to help determine from the outset which children are more likely to have strangulated bowel so that urgent surgery can be performed.

Acute intussusception results in local inflammation and vascular congestion. Activation of leucocytes causes the release of inflammatory mediators. Inflammatory cytokines promote leucocyte adhesion and migration through post-capillary vascular endothelium, and contribute to microvascular disturbances leading to hypovolaemia and bacterial translocation through the gut wall<sup>7</sup>. Neopterin (D-erythro-6-(1',2',3'-trihydroxypropyl)pterin) is a metabolite of guanosine triphosphate and is secreted in large quantities by activated macrophages under stimulation by interferon  $\gamma$ <sup>8</sup>. Increased plasma neopterin concentrations have been found to correlate with disease severity in acute pancreatitis and with death in critically ill adult patients<sup>9,10</sup>. Significantly raised levels of plasma IL-6 and neopterin were detected in these children with acute intussusception, particularly in those who needed operative intervention, suggesting that plasma concentrations of these cytokines may be linked to disease severity. TNF- $\alpha$  concentrations, although increased, were similar in acute and convalescent samples. However, TNF- $\alpha$  has a short circulating half-life<sup>11</sup> and an early but transient release of this cytokine in acute intussusception might have been missed.

Plasma CRP concentration was significantly raised at diagnosis in acute intussusception, with a direct correlation to disease severity, and proved to be the best marker in this study. Plasma CRP is easily measured and routinely available in most laboratories. It deserves further investigation as a potentially valuable index of disease severity at the time of diagnosis of intussusception. Measurement of CRP may predict children with an irreducible intussusception with vascular compromise.

There were no significant differences between acute and convalescent measurements of plasma MDA, a circulating marker of lipid peroxidation and free radical activity. Free radical damage may well be important at a local level in intussusception or specifically in ischaemia-reperfusion injury but further investigations are needed to define the potential therapeutic role of free radical scavengers such as mannitol or allopurinol in children with acute intussusception.

Endotoxin, the lipopolysaccharide component of the cell wall of Gram-negative bacteria is toxic to humans in nanogram quantities. Lipid A is the main toxic moiety of endotoxin and is responsible for many of the pathophysiological responses leading to multiple organ failure in

Gram-negative sepsis<sup>12</sup>. Although gut translocation of Gram-negative bacteria in intussusception was recognized many years ago<sup>13</sup>, the role of endotoxin in this condition has been neglected. Previous investigation appears to have been limited to a mouse model of endotoxin-induced intussusception<sup>14,15</sup>. In the present study, all blood cultures were sterile but there was evidence of endotoxaemia, particularly in children who needed intestinal resection. Endotoxaemia may be an important factor influencing morbidity in intussusception.

IgG EndoCAbs are present at birth, probably as a result of maternal (transplacental) transmission<sup>16</sup>. Levels gradually decline over the first 3 months of life after which endogenous IgG EndoCAb begins to increase, reaching adult concentrations at about 6–7 years of age<sup>5</sup>. IgM EndoCAb (endogenous) is virtually absent in the first month of life but increases gradually to around adult levels by 1 year of age. Endotoxaemia leads to an initial depletion in both IgG and IgM EndoCAb concentrations. Subsequently, the humoral anamnestic EndoCAb response may be triggered, and EndoCAb levels can rise rapidly<sup>5</sup>. The antibody responses observed in the children in the present study may indicate endotoxin exposure even where endotoxaemia could not be measured. Acute IgM and IgG EndoCAb levels were relatively normal but IgM levels were significantly increased in convalescent sera. The time course of the endotoxin antibody response in adults, with IgM concentrations peaking around day 5–7 and IgG peaking around day 7–9, would be compatible with an endotoxin antibody response in children following acute intussusception, since convalescent samples were taken from most patients 2–6 days after the acute samples. The observation that depressed EndoCAb is associated with a poor clinical outcome in other clinical conditions<sup>17–20</sup> is compatible with the observation that children who required intestinal resection had the lowest acute IgM EndoCAb levels.

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