



Production of lymphocyte-derived cytokines by whole umbilical cord blood cultures stimulated with mitogens and allergens

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Abstract

A whole blood culture technique was used to establish conditions for stimulating the production of cytokines by cord blood lymphocytes. The cultures were stimulated with mitogens (concanavalin A and phytohaemagglutinin) and allergens (beta-lactoglobulin (BLG), ovalbumin (OVA) and house dust mite (*Der p 1*)) at a range of concentrations. Interleukin (IL-) 2, IL-4, IL-10, IL-13 and interferon-gamma (IFN- γ) concentrations were assayed in the supernatants at 24, 48 and 72 h. Stimulation with mitogens but not allergens induced increases in IL-2 and IL-13 concentrations. IFN- γ was strongly induced by mitogenic stimulation: maximal responses were seen at 48 h. Stimulation with the allergens also induced an increase in IFN- γ concentration which was maximal for 100 μ g/ml of BLG and OVA. *Der p 1* induced the highest IFN- γ production among the allergens. IL-4 concentrations were increased in mitogen and *Der p 1* stimulated cultures. This was maximal at 48 and 24 h, respectively. IL-10 was induced with mitogen and allergen stimulation. Thus, this study has established conditions for assessing production of lymphocyte-derived cytokines in a simple whole umbilical cord blood culture system.

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1. Introduction

Interferon-gamma (IFN- γ) is produced by T helper 1 (Th1)-type lymphocytes and downregulates the immunoglobulin E (IgE) response to antigen exposure [1–4]. Conversely, Th2-type cells produce interleukin (IL)-4 and IL-13, which upregulate IgE production, and IL-10, which suppresses the development of Th1 cells [5,6]. A bias of the T helper phenotype towards Th2 is thought to favour the development of atopic disease [7]. There is emerging evidence that an imbalance in the ratio of production of Th1 and Th2 cytokines is present in early life and perhaps exists antenatally [8]. Furthermore, several groups have linked the pattern of cytokine production by human umbilical cord blood with risk of

later development of atopic disease. For example, neonates with the lowest cord blood IFN- γ production are at higher risk for subsequent development of allergic disease [9–12]. Thus, there is increasing interest in measuring cytokine production by cord blood cells and in applying such measures to large epidemiological or intervention studies. Studies investigating cord blood lymphocyte-derived cytokines have often used cord blood mononuclear cells (CBMCs) isolated by density gradient centrifugation. This is a time consuming and expensive procedure that may not be easily applied to field studies or to very large numbers of samples. Also, standard Ficoll-Hypaque separation techniques give poor CBMC purity, due to the presence of nucleated red blood cells [13]. The inclusion of a proportion of nucleated red cells results in inaccurate CBMC counts prior to culture. Although this can be improved by further rounds of Ficoll-Hypaque separation, this may be at the cost of adverse effects such as increased apoptosis and altered cytokine production [14]. Finally,

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Table 1

Interleukin 2 concentration (U/ml) in the supernatants of diluted umbilical cord blood cultured for different periods in the presence of mitogens

Mitogen ($\mu\text{g/ml}$)	24 h		48 h		72 h	
	1:5	1:10	1:5	1:10	1:5	1:10
None	0.9 $\pm$ 0.1a	0.9 $\pm$ 0.1a	1.0 $\pm$ 0.2a	1.0 $\pm$ 0.1a	1.0 $\pm$ 0.1a	0.9 $\pm$ 0.1a
Con A (5)	11.0 $\pm$ 3.6b	5.4 $\pm$ 2.9b	3.4 $\pm$ 0.9b‡	3.1 $\pm$ 1.1b	1.2 $\pm$ 0.2ab‡	1.0 $\pm$ 0.1a‡
Con A (10)	18.4 $\pm$ 3.5b	6.3 $\pm$ 2.4b*	5.6 $\pm$ 1.5b‡	2.4 $\pm$ 0.5ab	1.6 $\pm$ 0.3ab‡¶	1.0 $\pm$ 0.1a‡
Con A (20)	29.7 $\pm$ 4.2c	5.4 $\pm$ 1.2b*	13.1 $\pm$ 3.4c‡	2.2 $\pm$ 0.5ab*‡	2.9 $\pm$ 0.9b‡¶	1.0 $\pm$ 0.1a‡
Con A (50)	28.4 $\pm$ 4.9c	2.6 $\pm$ 0.7ab*	13.3 $\pm$ 5.9c‡	1.9 $\pm$ 0.6ab*	3.4 $\pm$ 1.5b‡¶	1.0 $\pm$ 0.2a*‡
Con A (100)	19.3 $\pm$ 4.5bc	1.5 $\pm$ 0.2a*	4.3 $\pm$ 0.6b‡	1.5 $\pm$ 0.2ab*	1.4 $\pm$ 0.1ab‡¶	1.2 $\pm$ 0.2a
PHA (2.5)	2.2 $\pm$ 0.8ab	2.2 $\pm$ 0.6ab	1.1 $\pm$ 0.1ab	1.6 $\pm$ 0.3ab	1.0 $\pm$ 0.1a‡	1.0 $\pm$ 0.1a
PHA (5)	11.5 $\pm$ 4.8c	2.5 $\pm$ 0.5ab*	2.1 $\pm$ 0.5ab‡	3.0 $\pm$ 0.7b	1.2 $\pm$ 0.1ab‡	1.1 $\pm$ 0.1a¶
PHA (10)	12.6 $\pm$ 3.1c	1.9 $\pm$ 0.3ab*	6.2 $\pm$ 1.0c‡	1.8 $\pm$ 0.3ab*	2.4 $\pm$ 0.8b‡¶	1.0 $\pm$ 0.1a*‡
PHA (20)	6.3 $\pm$ 1.4b	1.7 $\pm$ 0.6ab*	3.7 $\pm$ 0.8bc	1.6 $\pm$ 0.5ab	1.5 $\pm$ 0.3ab‡	1.1 $\pm$ 0.1a
PHA (50)	5.5 $\pm$ 2.8b	3.0 $\pm$ 1.6b	3.5 $\pm$ 1.8bc	2.1 $\pm$ 0.8ab	1.7 $\pm$ 0.4ab‡	1.2 $\pm$ 0.1a‡

Data are mean $\pm$ SEM ($n = 4$). Values for each mitogen down a column not sharing a letter are significantly different (one-factor ANOVA). ‡, † and ¶ indicate 48 h is different from 24 h, 72 h is different from 24 h and 72 h is different from 48 h, respectively (one-factor ANOVA). * indicates 1:10 dilution is different from 1:5 dilution (Student's paired t -test).

the separation of mononuclear cells can effect the ratio of different cytokine producing cells compared with the ratios present in whole blood [15–17]. For these reasons it seems desirable to establish a simple technique involving culture of whole blood that is rapid, repeatable, less likely to impair/alter subsequent cytokine production and easily transferred to field conditions or to situations involving large numbers but small volumes of cord blood samples. Thus, this study set out to establish conditions to investigate cytokine responses of whole cord blood to mitogens (concanavalin A (Con A) and phytohaemagglutinin (PHA)) and allergens (beta-lactoglobulin (BLG), ovalbumin (OVA) and house dust mite (*Dermatophagoides pteronyssinus*) allergen (*Der p 1*)). Cytokines measured were the Th1-type cytokines IL-2 and IFN- γ and the Th2-type cytokines IL-4, IL-13 and IL-10.

2. Results

2.1. Interleukin 2

There were significant effects of mitogen concentration, time in culture, and blood dilution (all $P < 0.001$ for both Con A and PHA) on IL-2 concentration. For both mitogens and at all three time points in culture, IL-2 concentration was higher for 1:5 than 1:10 diluted blood, although this difference was less apparent at 72 h (Table 1). There was a 'bell-shaped' curve of IL-2 response to increasing concentrations of both mitogens, although this was less apparent for the 1:10 diluted blood at the longer culture periods (Table 1). The highest IL-2 concentrations occurred at 24 h of culture (33-fold and 14-fold increases from the unstimulated control for Con A and PHA, respectively) (Table 1). The maximal concentration of IL-2 was achieved in the 1:5 diluted blood culture stimulated with either 20 or 50 $\mu\text{g/ml}$ Con

A or 5 or 10 $\mu\text{g/ml}$ PHA (Table 1). IL-2 production was not induced in response to BLG, OVA or *Der p 1*.

2.2. Interferon-gamma

There was a significant effect of mitogen concentration ($P = 0.046$ for Con A and $P = 0.006$ for PHA) and time in culture ($P = 0.025$ for Con A only), but not of blood dilution, on IFN- γ concentration. When Con A was used as mitogen, IFN- γ concentration tended to be higher for the 1:5 than the 1:10 diluted blood at all three time points, but this difference did not become significant (Table 2). When PHA was used as mitogen, IFN- γ concentration was similar for the 1:5 and 1:10 diluted blood at each time point (Table 2). There was a bell-shaped curve of IFN- γ response to increasing concentrations of both mitogens when 1:5 diluted blood was used (Table 2). The highest IFN- γ concentrations occurred at 48 h of culture (14-fold and 9-fold increases from the unstimulated control for Con A and PHA, respectively) (Table 2). The maximal concentration of IFN- γ was achieved in the 1:5 diluted blood culture stimulated with either 10–50 $\mu\text{g/ml}$ Con A or 10 $\mu\text{g/ml}$ PHA (Table 2).

Most blood samples produced IFN- γ in response to BLG and OVA (Fig. 1). There were significant effects of BLG and OVA concentration ($P < 0.001$ at each time point for both BLG and OVA) on IFN- γ concentration. In all cases, the concentration of IFN- γ was significantly higher in the presence of allergen than in cultures incubated without allergen. IFN- γ concentration increased with increasing concentration of BLG and OVA (Fig. 1) and was not significantly different between the 1:5 and the 1:10 diluted blood (data not shown). For a particular concentration of BLG or OVA, the concentration of IFN- γ was not significantly different at the different time points of culture.

Stimulation with 10 $\mu\text{g/ml}$ *Der p 1* induced IFN- γ production by all blood samples (Fig. 2), and the

Table 2

Interferon-gamma concentration (IU/ml) in the supernatants of diluted umbilical cord blood cultured for different periods in the presence of mitogens

Mitogen ($\mu\text{g/ml}$)	24 h		48 h		72 h	
	1 : 5	1 : 10	1 : 5	1 : 10	1 : 5	1 : 10
None	26 $\pm$ 25a	36 $\pm$ 27a	41 $\pm$ 29a	27 $\pm$ 14a	39 $\pm$ 34a	30 $\pm$ 27a
Con A (5)	89 $\pm$ 45ab	78 $\pm$ 35a	163 $\pm$ 85ab	168 $\pm$ 24b	138 $\pm$ 67ab	208 $\pm$ 77b
Con A (10)	99 $\pm$ 59ab	75 $\pm$ 31a	457 $\pm$ 355ab	180 $\pm$ 46b	418 $\pm$ 293b	272 $\pm$ 135ab
Con A (20)	103 $\pm$ 45b	65 $\pm$ 25a	401 $\pm$ 240b	164 $\pm$ 69b	393 $\pm$ 220b	479 $\pm$ 388ab
Con A (50)	90 $\pm$ 56ab	55 $\pm$ 19a	578 $\pm$ 453ab	105 $\pm$ 42b	300 $\pm$ 161b	114 $\pm$ 57ab
Con A (100)	51 $\pm$ 23ab	68 $\pm$ 29a	121 $\pm$ 66a	101 $\pm$ 35b	147 $\pm$ 82ab	79 $\pm$ 27ab
PHA (2.5)	65 $\pm$ 25ab	87 $\pm$ 32a	62 $\pm$ 21ab	178 $\pm$ 55a	59 $\pm$ 27a	190 $\pm$ 73a
PHA (5)	112 $\pm$ 46b	129 $\pm$ 55a	137 $\pm$ 31b	205 $\pm$ 80a	140 $\pm$ 45a	160 $\pm$ 67a
PHA (10)	148 $\pm$ 84b	105 $\pm$ 65a	367 $\pm$ 264b	138 $\pm$ 69a	164 $\pm$ 93a	123 $\pm$ 86a
PHA (20)	110 $\pm$ 64ab	79 $\pm$ 39a	137 $\pm$ 86ab	112 $\pm$ 41a	85 $\pm$ 50a	86 $\pm$ 26a
PHA (50)	58 $\pm$ 22ab	60 $\pm$ 38a	71 $\pm$ 33ab	87 $\pm$ 29a	61 $\pm$ 1a	87 $\pm$ 22a

Data are mean $\pm$ SEM ($n = 4$). Values for each mitogen down a column not sharing a letter are significantly different (one-factor ANOVA).

concentration of IFN- γ was significantly higher in the presence of *Der p 1* ($P = 0.012$ at all time points in both blood dilutions). IFN- γ concentration did not differ between 1 : 5 and 1 : 10 diluted blood and did not differ between time points of culture (Fig. 2).

2.3. Interleukin 4

There were significant effects of mitogen concentration, time in culture, and blood dilution (all $P < 0.001$ for both Con A and PHA) on IL-4 concentration. For both mitogens and at all three time points in culture, IL-4 concentration tended to be higher for 1 : 5 than 1 : 10 diluted blood (Table 3). There was a bell-shaped curve of IL-4 response to increasing concentration of Con A, whereas the response to increasing concentration of PHA was more linear (Table 3). The highest IL-4 concentrations occurred at 48 h of culture (approximately 33-fold increase from the unstimulated control for both mitogens) (Table 3). The maximal concentration of IL-4 was achieved in the 1 : 5 diluted blood culture stimulated with either 50 $\mu\text{g/ml}$ Con A or PHA (Table 3).

IL-4 production was not induced in response to BLG or OVA. In contrast, *Der p 1* induced IL-4 production (Table 3), such that the concentration of IL-4 was significantly higher in the presence of *Der p 1* at 24 and 48 h (Table 3). The concentration of IL-4 was highest at 24 h of culture in the 1 : 5 diluted blood (Table 3). This concentration was significantly higher than in the 1 : 10 diluted blood at the same time point ($P = 0.046$) and was significantly higher than at 72 h of culture ($P = 0.034$).

2.4. Interleukin 13

There were significant effects of mitogen concentration, time in culture, and blood dilution (all $P < 0.001$ for both Con A and PHA) on IL-13 concentration. IL-13 concentrations in the cultures stimulated with the

higher concentrations of Con A were higher for 1 : 5 than 1 : 10 diluted blood at all three time points (Table 4). In contrast, IL-13 production in response to PHA did not differ significantly between the two blood dilutions (Table 4). The IL-13 response to increasing concentrations of Con A for 1 : 5 diluted blood and of PHA for both blood dilutions was linear, whereas for Con A in the 1 : 10 dilution of blood it was bell-shaped (Table 4). The highest IL-13 concentrations occurred at 72 h of culture (Table 4). The maximal concentration of IL-13 was achieved in the 1 : 5 diluted blood culture stimulated with either 100 $\mu\text{g/ml}$ Con A or the 1 : 10 diluted blood culture stimulated with 50 $\mu\text{g/ml}$ PHA (Table 3). IL-13 production was not induced in response to BLG, OVA or *Der p 1*.

2.5. Interleukin 10

There was a significant effect of mitogen concentration ($P < 0.001$ for both Con A and PHA) and time in culture ($P = 0.003$ for Con A and $P = 0.001$ for PHA), but not of blood dilution, on IL-10 concentration. There was a bell-shaped curve of IL-10 response to increasing concentrations of Con A and PHA (Table 5). The highest IL-10 concentrations occurred at 48 h of culture (10-fold and 44-fold increases from the unstimulated control for Con A and PHA, respectively) (Table 5). The maximal concentration of IL-10 was achieved in the 1 : 5 diluted blood culture stimulated with either 50 $\mu\text{g/ml}$ Con A or 20 $\mu\text{g/ml}$ PHA (Table 5).

Most blood samples produced IL-10 in response to BLG and OVA (Fig. 3). There were significant effects of BLG and OVA concentration ($P < 0.001$ at each time point for both BLG and OVA) on IL-10 concentration. In all cases, the concentration of IL-10 was significantly higher in the presence of allergen than in cultures incubated without allergen. IL-10 concentration increased with increasing concentration of these allergens (Fig. 3) and was not significantly different between the

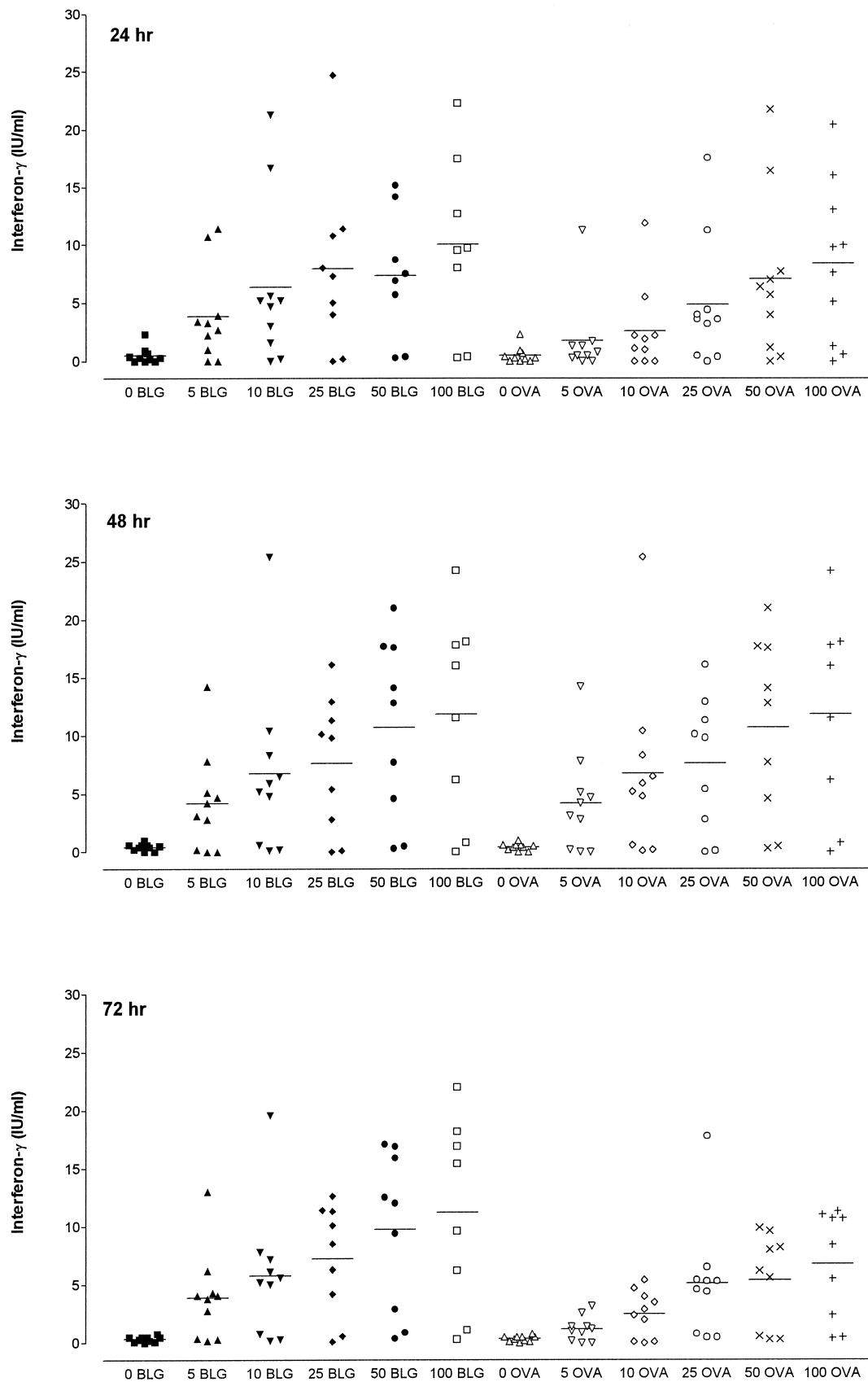


Fig. 1. Interferon-gamma concentrations (IU/ml) in beta-lactoglobulin (BLG; 0, 5, 10, 25, 50, 100 μ g/ml) and ovalbumin (OVA; 0, 5, 10, 25, 50, 100 μ g/ml) stimulated 1:5 diluted whole cord blood cultures at 24, 48 and 72 h. Data are for 8–10 samples.

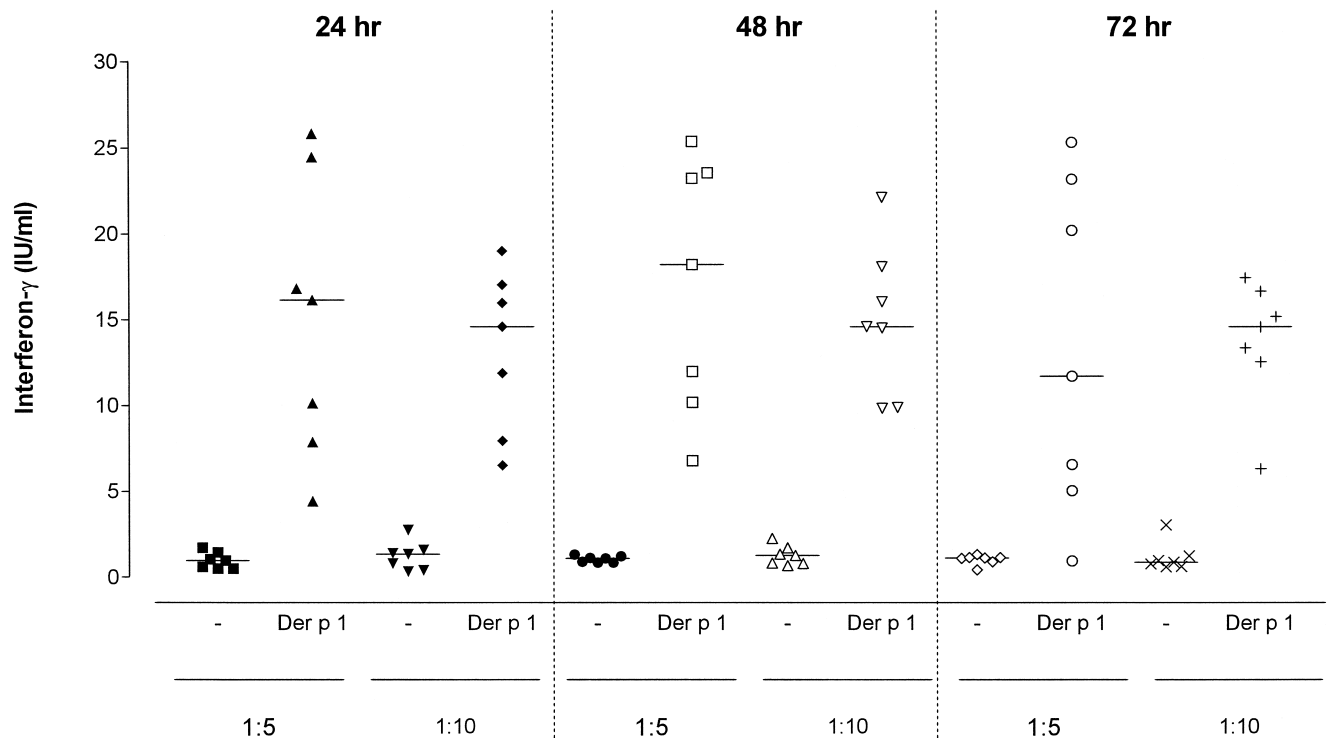


Fig. 2. Interferon-gamma concentrations (IU/ml) in 1:5 and 1:10 diluted whole cord blood cultures at 24, 48 and 72 h stimulated with 10 µg/ml *Der p 1* allergen. Data are for 7–10 samples.

1:5 and the 1:10 diluted blood (data not shown). At each concentration of BLG, the concentration of IL-10 was significantly lower at 48 h than at 24 h and was significantly lower at 72 h than at both 24 and 48 h ($P < 0.004$ for 1:5 diluted blood and $P < 0.008$ for 1:10 diluted blood). At OVA concentrations of 25, 50 and 100 µg/ml, the concentration of IL-10 was significantly

lower at 48 h than at 24 h ($P < 0.05$). At each concentration of OVA, the concentration of IL-10 was significantly lower at 72 h than at both 24 and 48 h ($P < 0.037$ for 1:5 diluted blood and $P < 0.038$ for 1:10 diluted blood). Thus, for both these allergens the highest concentrations of IL-10 were seen after 24 h of culture (Fig. 3).

Table 3

Interleukin 4 concentration (pg/ml) in the supernatants of diluted umbilical cord blood cultured for different periods in the presence of mitogens and *Der p 1*

Stimulant (µg/ml)	24 h		48 h		72 h	
	1:5	1:10	1:5	1:10	1:5	1:10
None	0.1 ± 0.1a	0.2 ± 0.1a	0.2 ± 0.1a	0.3 ± 0.2a	0.1 ± 0.1a	0.1 ± 0.1a
Con A (5)	1.3 ± 0.6a	1.2 ± 0.4b	1.9 ± 1.0ab	2.5 ± 1.2ab	0.5 ± 0.2a	1.2 ± 0.8b
Con A (10)	1.3 ± 0.4a	1.8 ± 0.7b	2.4 ± 1.1ab	3.1 ± 1.0b	0.9 ± 0.2b	0.7 ± 0.1ab
Con A (20)	1.7 ± 0.7ab	1.6 ± 0.7b	3.8 ± 0.8bc	2.7 ± 0.9b	2.2 ± 0.7bc	0.9 ± 0.8b
Con A (50)	3.4 ± 1.2b	0.5 ± 0.2a*	7.1 ± 3.0c	2.0 ± 1.2ab	3.9 ± 1.4c	0.7 ± 0.7ab
Con A (100)	2.5 ± 0.6b	0.3 ± 0.1a*	3.3 ± 1.7bc	0.4 ± 0.2a*	1.6 ± 0.7b	0.3 ± 0.2ab*
PHA (2.5)	1.8 ± 0.9a	1.8 ± 0.8b	0.3 ± 0.2a	1.8 ± 0.6ab	0.1 ± 0.1a	0.1 ± 0.0a
PHA (5)	4.0 ± 2.0b	1.9 ± 0.7b	2.0 ± 0.9b	3.5 ± 1.2b	0.9 ± 0.1b	0.7 ± 0.2b
PHA (10)	3.9 ± 1.6b	2.0 ± 0.6b	4.7 ± 1.6bc	3.1 ± 1.4b	2.9 ± 1.0b	0.9 ± 0.5b
PHA (20)	3.2 ± 0.9b	1.6 ± 0.3b	6.5 ± 1.7bc	3.0 ± 1.4b	3.0 ± 1.1b	1.2 ± 0.5c
PHA (50)	5.3 ± 1.0b	5.0 ± 0.9b	7.3 ± 1.8c	4.6 ± 1.9b	5.7 ± 1.7b	2.7 ± 1.1c
None	0.5 (0.2, 0.6)a	0 (0, 0)a	0.6 (0.4, 0.9)a	0 (0, 0.6)a	0.6 (0.1, 0.8)a	0 (0, 0.4)a
<i>Der p 1</i> (10)	1.3 (0.7, 3.0)b	0.6 (0.5, 0.8)b*	0.8 (0.5, 1.9)b	0.6 (0.1, 1.3)b	0.5 (0.1, 0.8)a†	0.4 (0.1, 0.6)a

Data are mean ± SEM for mitogens ($n = 4$) and median (interquartile range) for *Der p 1* ($n = 10$). Values for each stimulant down a column not sharing a letter are significantly different (one-factor ANOVA for Con A and PHA; Wilcoxon matched pairs test for *Der p 1*). † indicates 72 h is different from 24 h (Wilcoxon matched pairs test). * indicates 1:10 dilution is different from 1:5 dilution (Student's paired t -test or Wilcoxon matched pairs test).

Table 4

Interleukin 13 concentration (pg/ml) in the supernatants of diluted umbilical cord blood cultured for different periods in the presence of mitogens

Mitogen (µg/ml)	24 h		48 h		72 h	
	1:5	1:10	1:5	1:10	1:5	1:10
None	0.2 ± 0.2a	3.9 ± 3.1a	9.3 ± 9.3a	2.2 ± 2.2a	0.0 ± 0.0a	0.0 ± 0.0a
Con A (5)	12.2 ± 6.4b	6.9 ± 3.0a*	47.0 ± 9.8b‡	88.2 ± 38.9a	47.6 ± 12.3b‡	153.1 ± 76.3b‡
Con A (10)	16.1 ± 10.4b	9.8 ± 3.9a	90.9 ± 14.0b‡	204.8 ± 89.5ab‡	120.3 ± 36.4b‡	244.8 ± 117.0bc*‡
Con A (20)	30.9 ± 8.8b	40.4 ± 24.6ab	205.6 ± 36.6c‡	405.8 ± 157.0b‡	270.3 ± 60.9b‡	511.8 ± 131.2c‡
Con A (50)	151.4 ± 41.3c	48.3 ± 17.3b	1144.6 ± 457.7d‡	345.0 ± 96.7b‡	1517.6 ± 465.1c‡	383.7 ± 177.1c*‡
Con A (100)	327.7 ± 67.0d	32.1 ± 8.2b*	1679.4 ± 416.4d‡	159.5 ± 79.1ab*‡	1875.3 ± 606.0c‡	238.9 ± 117.5bc*‡
PHA (2.5)	4.5 ± 2.6b	7.3 ± 4.0a	9.8 ± 4.3a	26.8 ± 3.9b‡	9.6 ± 8.1b	36.8 ± 2.8b‡
PHA (5)	9.9 ± 4.7b	20.2 ± 7.7b	49.4 ± 12.7b‡	53.2 ± 26.8b	50.9 ± 25.2bc‡	78.6 ± 25.4b
PHA (10)	22.7 ± 10.1b	17.9 ± 7.4b	93.0 ± 21.6bc‡	46.5 ± 34.3b	108.4 ± 20.5c‡	62.0 ± 33.9b
PHA (20)	23.8 ± 15.8b	14.5 ± 9.4b	68.3 ± 46.9bc	83.0 ± 30.5b	82.5 ± 23.5bc	147.8 ± 71.2c‡
PHA (50)	43.7 ± 31.3b	43.0 ± 33.3b	166.3 ± 80.6c	362.6 ± 140.8c‡	431.0 ± 149.7d‡	1536.2 ± 586.8d‡

Data are mean ± SEM ($n = 4$). Values for each mitogen down a column not sharing a letter are significantly different (one-factor ANOVA). ‡ indicate 48 h is different from 24 h, and 72 h is different from 24 h, respectively (one-factor ANOVA). * indicates 1:10 dilution is different from 1:5 dilution (Student's paired t -test).

Stimulation with 10 µg/ml *Der p 1* induced IL-10 production by all blood samples (Fig. 4), and the concentration of IL-10 was significantly higher in the presence of *Der p 1* ($P = 0.012$ at all time points in both blood dilutions). IL-10 concentration did not differ between 1:5 and 1:10 diluted blood (Fig. 4). However, IL-10 concentration was significantly lower at 48 h than at 24 h and was significantly lower at 72 h than at both 24 and 48 h ($P < 0.001$ for 1:5 diluted blood and $P = 0.001$ for 1:10 diluted blood) (Fig. 4).

3. Discussion

The production of Th1-type and Th2-type cytokines by isolated CBMCs has been demonstrated previously after stimulation with mitogens [18–22] and allergens [8,12,22]. The current study establishes conditions for using a whole blood culture technique to assess cytokine production by cord blood lymphocytes. Stimulation

with mitogens (Con A and PHA) and allergens (BLG, OVA and *Der p 1*) was compared and the production of a range of cytokines by Th1-type cells (IL-2 and IFN- γ) and Th2-type cells (IL-4, IL-13 and IL-10) was investigated. The study shows that diluted whole umbilical cord blood cultures can be induced with mitogens to increase IL-2, IFN- γ , IL-4, IL-13 and IL-10 production. The levels of production of these cytokines were different according to whether Con A or PHA was used: Con A induced higher production of IL-2, IFN- γ and IL-13 (except at 72 h culture), whereas PHA induced higher production of IL-10. This may reflect differences in the cell signalling processes induced by the mitogens that lead to expression of the specific cytokine genes. All three allergens induced significant production of IFN- γ and IL-10, while only *Der p 1* induced IL-4 production, and then only at a low level. Concentrations of these cytokines were higher after mitogen than after allergen stimulation, simply reflecting the fact that most T cells present in the culture will respond to the

Table 5

Interleukin 10 concentration (pg/ml) in the supernatants of diluted umbilical cord blood cultured for different periods in the presence of mitogens

Mitogen (µg/ml)	24 h		48 h		72 h	
	1:5	1:10	1:5	1:10	1:5	1:10
None	81 ± 53a	115 ± 78a	32 ± 15a	61 ± 42a	11 ± 4a	30 ± 13a
Con A (5)	155 ± 52ab	153 ± 61a	165 ± 37ab	240 ± 80ab	81 ± 39ab	182 ± 73ab
Con A (10)	187 ± 41ab	142 ± 33a	237 ± 113b	354 ± 123b	103 ± 52ab	276 ± 129b
Con A (20)	250 ± 60b	133 ± 31a	308 ± 158b	309 ± 83b	120 ± 47b	183 ± 49ab
Con A (50)	252 ± 32b	41 ± 12a	340 ± 80b	80 ± 26a	124 ± 12b¶	83 ± 40ab
Con A (100)	177 ± 52ab	28 ± 10a	250 ± 51b	33 ± 9a	126 ± 43b	33 ± 12a
PHA (2.5)	190 ± 37a	301 ± 156a	294 ± 139b	690 ± 326b	93 ± 43b	521 ± 300b
PHA (5)	326 ± 59b	317 ± 151a	836 ± 471b	998 ± 399b	289 ± 174b	1100 ± 539c
PHA (10)	115 ± 78a	192 ± 25a	1421 ± 421c	1055 ± 442b	830 ± 358c	1233 ± 666c
PHA (20)	153 ± 61a	187 ± 31a	1511 ± 114c	633 ± 426b	1126 ± 394c	866 ± 650bc
PHA (50)	142 ± 33a	199 ± 18a	396 ± 70b	300 ± 244b	717 ± 342c	355 ± 246b

Data are mean ± SEM ($n = 4$). Values for each mitogen down a column not sharing a letter are significantly different (one-factor ANOVA). ¶ indicates 72 h is different from 48 h (one-factor ANOVA).

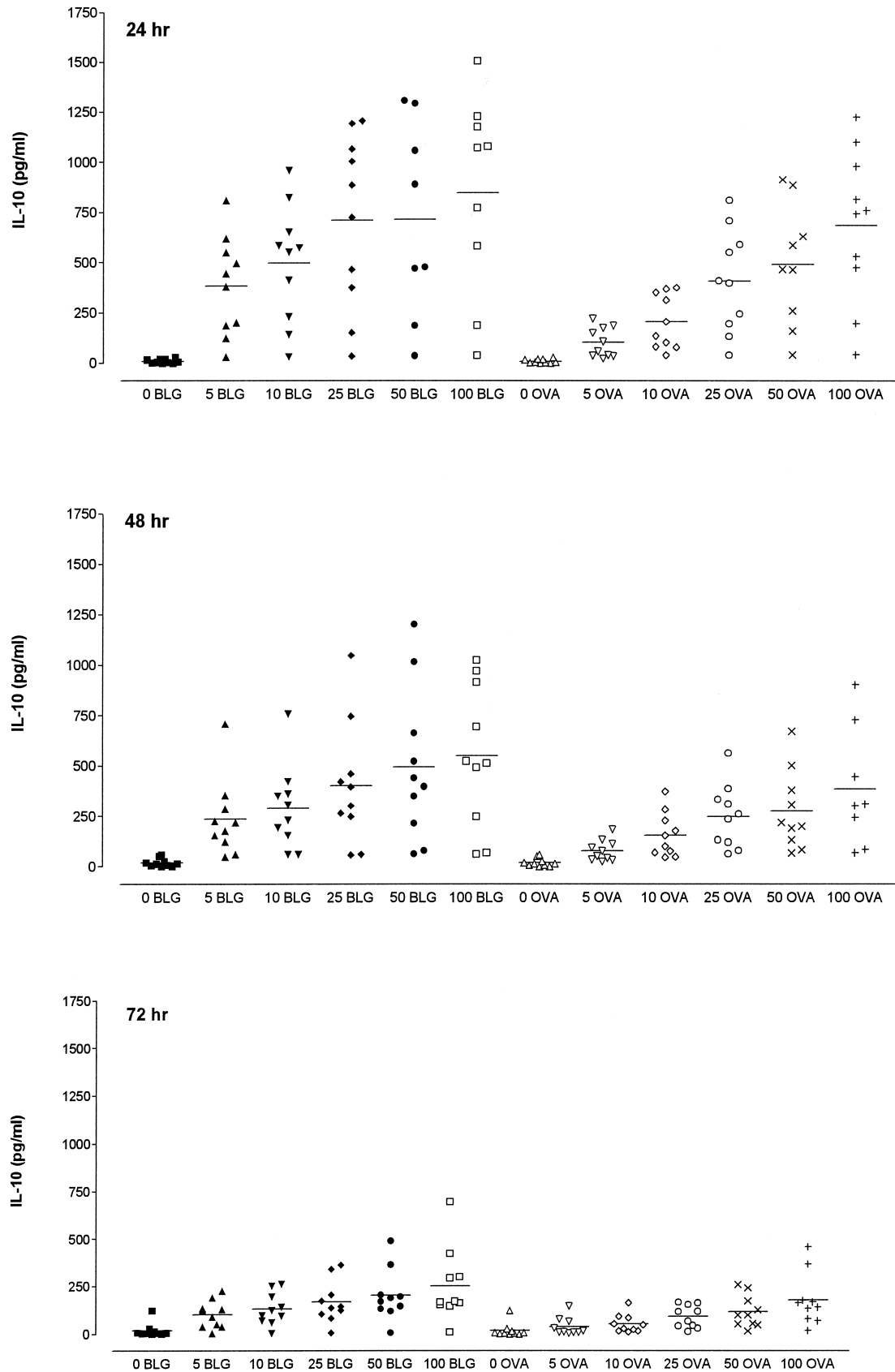


Fig. 3. Interleukin 10 concentrations (pg/ml) in beta-lactoglobulin (BLG; 0, 5, 10, 25, 50, 100 µg/ml) and ovalbumin (OVA; 0, 5, 10, 25, 50, 100 µg/ml) stimulated 1:5 diluted whole cord blood cultures at 24, 48 and 72 h. Data are for 9–10 samples.

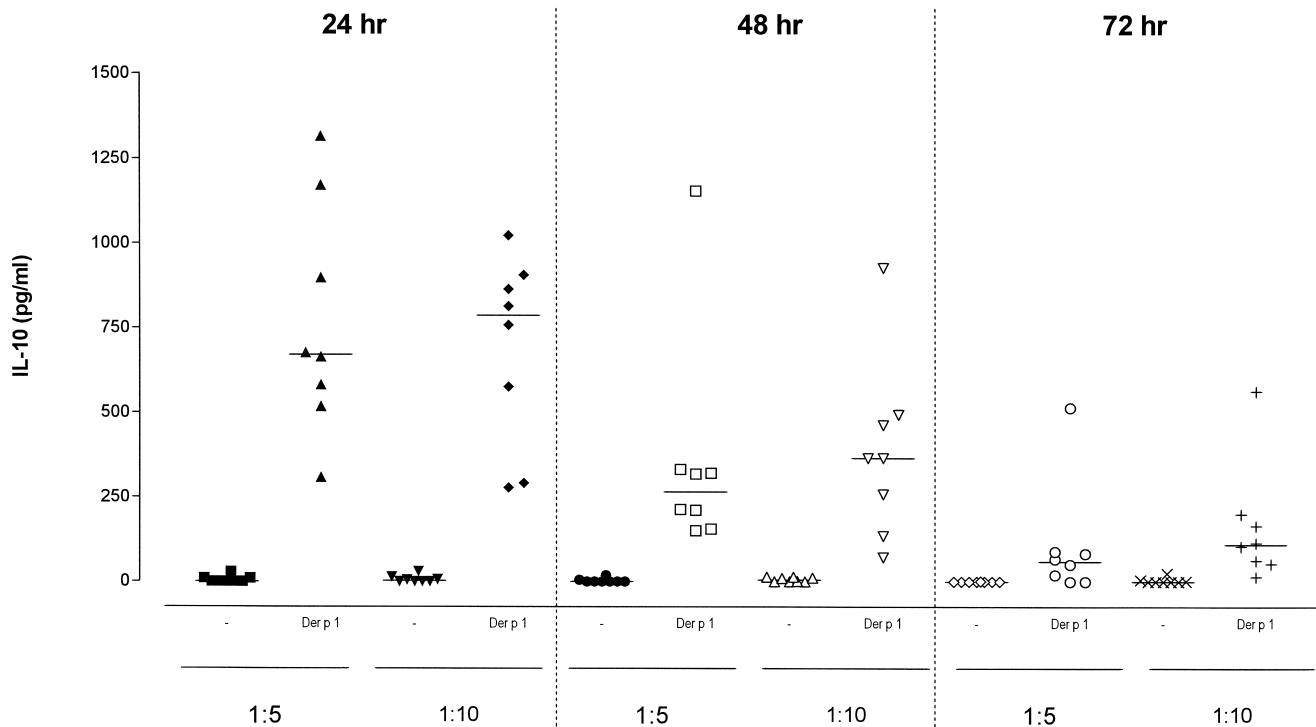


Fig. 4. Interleukin 10 concentrations (pg/ml) in 1:5 and 1:10 diluted whole cord blood cultures at 24, 48 and 72 h stimulated with 10 µg/ml *Der p 1* allergen. Data are for 8 samples.

mitogens, whereas only a limited number will be allergen specific. IL-2 production in response to stimulation through the CD3 signalling pathway is known to be lower in cord blood T cells when compared with those from adults [23]. However, the CD3 signalling pathway leading to IL-2 production is clearly intact in cord blood T cells since mitogens, which also act through CD3 [24], induce significant IL-2 (and other cytokine) production. Thus, the lack of induction of some cytokines by allergens must reflect differences in post-CD3 signalling pathways.

The early increase in the concentration of some cytokines followed by a decrease in their concentration over time (e.g. IL-2 in response to mitogens; IL-10 in response to allergens) suggests either that these cytokines are produced early and then degraded or are removed from the culture by binding to receptors and subsequent uptake. However, maximal IL-13 concentrations did not occur until 72 h of culture, which was after the maximal concentrations of the other cytokines, including IL-4, had occurred. It appears that IL-13 is produced by T cells in the absence of IL-4 or after IL-4 production has been down-regulated [25–27], with kinetics more like those of IFN- γ [25,26]. The pattern of production of cytokines in the current study is in accordance with these earlier reports.

Apoptosis has been demonstrated to occur in cultured diluted adult whole blood, although to a lesser

extent than in cultures of purified blood mononuclear cells [14]. Apoptosis was not monitored in the current study, but could contribute to the pattern of appearance of cytokines in cell culture supernatants in two ways. First, apoptosis would remove cytokine-producing cells from the culture. Secondly, apoptotic cells would release their cytosolic contents, including cytokines, into the culture medium.

In summary, this study demonstrates that a simple diluted whole blood culture technique can be used to measure mitogen- and allergen-induced cytokine responses by cord blood cells *in vitro*. This method has several advantages over the use of isolated CBMCs. It is quicker, cheaper, requires less laboratory training and equipment and appears to be more repeatable than the culture of isolated CBMCs. The method uses a smaller volume of cord blood per culture than is required if CBMCs are to be isolated and this allows for a greater number of replicate samples and also permits use when only small volumes of blood are available. These features suggest that this technique will be particularly suitable for investigations involving large numbers of samples, such as epidemiological studies and large intervention studies, and for investigations carried out in field settings. The technique also has the advantage of retaining all cellular and non-cellular components of the cord blood (plasma, red cells and granulocytes are discarded when CBMCs

are isolated). Furthermore, these components are retained in the ratios in which they existed in vivo, and so this culture system represents a more physiological milieu in which to study cell function. Additionally, the difficult questions of which type of serum or plasma to add to the cultures and its concentration are avoided, since the cells are cultured in the physiological concentration of autologous plasma. The whole blood technique has one disadvantage: the cell populations and cell numbers in the blood sample are unknown and thus the precise nature of the culture is not controlled. However, this is offset by the recognition that the significant contamination of CBMCs by nucleated red blood cells, even after density centrifugation, means that cell counts are often inaccurate for isolated CBMCs [13]. Furthermore, studies comparing cytokine production by cultured diluted adult whole blood and purified blood mononuclear cells have shown similar patterns of production of the cytokines investigated here [15,28].

The use of this technique will allow easier screening of larger groups of babies with regard to the Th1 vs. Th2 cytokine bias at birth and atopic outcome. Whether the use of this method will confirm the data from existing studies remains to be determined.

4. Materials and methods

4.1. Materials

RPMI 1640 culture medium (without glutamine) was purchased from Autogen Bioclear, Calne, UK. L-Glutamine, benzyl penicillin, streptomycin sulphate, Con A, PHA, bovine BLG and chicken egg OVA were purchased from Sigma Chemical Co., Poole, UK. Affinity purified *Der p 1* was purchased from Indoor Biotechnologies, Cardiff, UK. Cytokine ELISA kits were purchased from Biosource Europe, Nivelles, Belgium.

4.2. Subjects and blood samples

Ethical approval was obtained from the Southampton and South West Hampshire Joint Local Research Ethical Committee. Umbilical vein blood from elective caesarean deliveries was taken into lithium heparin tubes. All deliveries were at term (40 ± 2 weeks) following uncomplicated pregnancies.

4.3. Whole cord blood culture

Whole cord blood was diluted 1 in 5 or 1 in 10 with RPMI 1640 medium supplemented with 0.75 mM L-glutamine and antibiotics (0.05 µg/ml each of benzyl penicillin and streptomycin sulphate). The diluted blood was placed in 24 well culture plates (2 ml/well) and

200 µl of stimulant was added. Final concentrations of stimulants were:

Con A 0, 5, 10, 20, 50 and 100 µg/ml
PHA 0, 2.5, 5, 10, 20 and 50 µg/ml
BLG 0, 5, 10, 20, 50, and 100 µg/ml
OVA 0, 5, 10, 20, 50, and 100 µg/ml
Der p 1 0 and 10 µg/ml.

Cultures were incubated for 24, 48 and 72 h at 37 °C in a humidified atmosphere with 5% CO₂. After incubation plates were centrifuged at 1000 rpm for 5 min and the supernatant medium aliquoted and stored at –20 °C for cytokine analysis.

4.4. Measurement of cytokine concentrations

IL-2, IL-4, IL-10, IL-13 and IFN-γ concentrations in culture supernatants were measured by ELISA. All ELISAs were carried out according to the manufacturer's instructions. Detection limits of the assays were 0.1 U/ml (IL-2), 0.27 pg/ml (IL-4), 1 pg/ml (IL-10), 12 pg/ml (IL-13), and 0.03 IU/ml (IFN-γ).

4.5. Statistics

Data were tested for normality using the Kolmogorov–Smirnov test. All data for Con A- or PHA-stimulated cultures were normally distributed and are presented as mean $\pm$ SEM. For these data, three-factor ANOVA was used to determine the significance of the effects of mitogen concentration, culture time and blood dilution. Where the effect of mitogen concentration was significant, one-factor ANOVA was used to determine differences between mitogen concentrations at each culture time and blood dilution. Where the effect of culture time was significant, one-factor ANOVA was used to determine differences between culture times at each mitogen concentration and blood dilution. Where the effect of blood dilution was significant, Student's paired *t*-test was used to determine differences between blood dilutions at each mitogen concentration and culture time. Some data for allergen-stimulated cultures were not normally distributed. Data for IL-4 concentrations after *Der p 1* stimulation are presented as median and interquartile range, while individual IFN-γ and IL-10 concentrations after allergen-stimulation and their median are presented. The effects of BLG or OVA concentration on the concentration of IFN-γ or IL-10 and of time of culture on the concentration of IFN-γ, IL-10 or IL-4 were determined by Friedman's test; subsequent pair-wise comparisons were made using the Wilcoxon matched pairs test. The effects of blood dilution and of *Der p 1* stimulation were determined using the Wilcoxon matched pairs test. All statistical analyses were performed using SPSS version 10 for Windows (SPSS Inc., Chicago, IL).

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