

Comparison of cytokine production in blood cell cultures of healthy children and adults

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The production of the cytokines IL-1- α , IL-1- β , IL-2, TNF- α , and IFN- γ was measured by a sensitive immunological assay in stimulated whole blood cell cultures from 52 healthy children (33 aged from 1 to 9 years and 19 aged between 10 and 17 years) and 67 healthy adults. When the higher absolute mononuclear cell counts in the peripheral blood samples of the children were taken into account, the relative production of all measured cytokines was lower in the cell cultures of the children than of the adults. In the group of the younger children (< 10 years) the differences were significant for all measured cytokines. In the group of older children ($\geq$ 10 years) the values were higher than in the younger children but lower than in adults. The findings indicate that the cellular immunological competence is or can be reduced in children and adolescents, particularly young children below 10 years of age. There seems to be a gradual development of cytokine production during childhood.

U. Elsässer-Beile¹, B. Dursunoglu¹, H. Gallati², J. Schulte Mönting³, S. von Kleist¹

¹ Institute of Immunobiology, University of Freiburg, Germany, ² Hoffmann-La Roche, CH-4002 Basel, Switzerland, ³ Institute of Medical Biometry, University of Freiburg, Germany

U. Elsässer-Beile, Institute of Immunobiology, Stefan-Meier-Str. 8, D-79104 Freiburg, Germany

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In view of new therapeutic modalities also in infants, it has become increasingly important to determine which components of the immune system are functionally immature at birth and when during childhood the immunological competence develops and reaches the adult capacity.

As a parameter for the immune status the capacity of peripheral leukocytes to produce cytokines may be taken. The current report focuses on the measurement of cytokines relevant for T-cell activation; i.e., IL-1- α , IL-1- β , IL-2, TNF- α , and IFN- γ produced in peripheral blood cell cultures after an initial triggering with a mitogenic stimulus in order to define the characteristic cytokine secretion profile of children at different ages. The aim of this study was to compare cytokine production in children of various ages with healthy adults.

Material and methods

Blood donors

Blood samples of 52 children (28 boys and 24 girls) were assayed. The children were grouped according to age into two groups, each of which covered a range of 9 and 8 years respectively and contained enough individuals for statistical calculations: group 1 with young children aged from 1 to 9 years (n=33)

and group 2 with older children aged from 10 to 17 years (n=19). The blood samples were collected by routine venipuncture in conjunction with other blood tests. There was no evidence of concurrent infections in these children. A protocol and consent form for obtaining blood from the children was reviewed by the local ethics commission.

Sixty-seven healthy adults (47 men and 20 women), aged between 20 and 59 years (mean 32 years), served as controls. A volume of 10 ml of heparinized blood was taken from each donor and the samples, kept at room temperature, were used within 3 h. A 0.5-ml aliquot was removed for total and differential leukocyte counts.

Whole blood cell cultures

Culturing was performed as previously described with a system for which optimal conditions and kinetics of cytokine production were established (1).

In brief, heparinized venous blood was diluted 1/10 with RPMI 1640 (Seromed, Berlin, Germany), which was supplemented with 50 U/ml penicillin (Seromed) and 50 mg/l streptomycin (Seromed) and distributed in 0.5-ml aliquots in 12-mm polystyrol tubes.

For mitogenic stimulation, phytohemagglutinin

(PHA; Wellcome, Burgwedel, Germany) at a final concentration of 10 mg/l or pokeweed mitogen (PWM; Sigma, Deisenhofen, Germany) at 0.5–5 mg/l was added. These mitogen concentrations had been shown to be optimal for cytokine induction.

Incubation of the cell cultures was performed at 37°C in a humidified atmosphere of 5% CO₂. After 4 days of culture without change of medium, 320 µl of supernatant was removed from each tube and assayed for cytokine levels.

Determination of cytokines

Enzyme-linked immunoassays (ELISA) were applied for qualitative and quantitative determinations of the cytokines (Hoffmann-La Roche, Basel). These tests were based on the sandwich principle and performed in one step. Since a strict correlation was found between the assay performance of naturally occurring cytokines and their recombinant counterparts, the latter were used as standards.

IFN-γ levels in the supernatants were determined as previously described (2). In brief, the IFN-γ containing supernatants and the samples for the IFN-γ standard curve were distributed together with a horseradish peroxidase-labeled monoclonal antibody to IFN-γ (clone 69) in microtiter plates previously coated with the same clone 69 monoclonal antibody against IFN-γ. After an incubation period of 24 h, unbound material was removed by a washing step and the amount of bound peroxidase (pod) was determined by a short incubation with tetramethylbenzidine. On stopping the reaction with sulfuric acid, the color changed to yellow and its intensity was determined at 450 nm by a computerized multi-channel photometer (Flow, Meckenheim, Germany). The amount of human IFN-γ was calculated from the standard curve, prepared with recombinant IFN-γ. This ELISA has an assay range of 50–1000 ng/l IFN-γ.

For determination of IL-1-α microtiter plates were coated with a polyclonal goat anti-human IL-1-α antibody. For the detection of protein bound IL-1-α, the pod-linked Fab-fragment of a polyclonal goat-anti-human-IL-1-α antibody was used. The standard was recombinant IL-1-α (assay range: 10–100 ng IL-1-α/l).

For the IL-1-β-ELISA the first immobilized antibody was a polyclonal goat anti-human-IL-1-β antibody. For the detection of bound IL-1-β, the pod-linked Fab-fragment of a polyclonal goat anti-human-IL-1-β antibody was used. The standard was prepared with recombinant IL-1-β. The test has an assay range of 100–1000 ng/l IL-1-β.

For determination of IL-2 microtiter plates were coated with two monoclonal mouse anti-human IL-2 antibodies (clone 3D5 and clone 7B1), and a third

pod-linked monoclonal mouse anti-human IL-2 antibody (clone 13A6) was used for detection of bound IL-2 (assay range: 50–1000 ng/l IL-2).

In the TNF-α ELISA the first immobilized antibody was a monoclonal mouse anti-human-TNF-α-antibody (clone 6b) and the second a pod-coupled polyclonal rabbit anti-human-TNF-α-antibody. Recombinant TNF-α was used as standard. The assay range was 20–500 ng/l TNF-α.

Determination of T-cell subsets by flow cytometry

Cells were stained with conjugated monoclonal antibodies of the Simultest™ reagents (Becton Dickinson, Heidelberg, Germany) and measured by flow cytometry using the SimulSET™ software (Becton Dickinson).

In brief, 100 µl of heparinized blood was mixed and incubated at 20°C with the appropriate amount of each Simultest™ reagent in separate tubes. Erythrocytes were then removed by lysis through the addition of FACS® Lysing Solution (Becton Dickinson) to the tubes. Samples were subsequently fixed with 1% formaldehyde (Riedel de Häen, Seelze, Germany).

Statistical analysis

The results in the patient and control groups were statistically evaluated using the analysis of variance, SAS® procedure GLM (SAS Institute, Inc, Cary, NC, USA). Due to the skewed distribution, all values were logarithmically transformed before this. In order to confirm the results the Kruskal-Wallis test (NPAR1 WAY) was applied to the untransformed values. However, it is not possible to consider more than one factor by means of nonparametric statistics.

Results

Differential white blood cell count and T-cell subpopulations

Both groups of children had significantly higher absolute lymphocyte and monocyte counts in their peripheral blood than adults (Table 1). Thus, also the absolute PBMC counts were higher in all children than in the adults. Lymphocyte subset ratios were similar in the blood samples of the children and adults except for the B cells, which were higher in the children. Median and quartile values of these data are given in Table 1. According to the different PBMC counts in the blood samples, the cytokine values in the whole blood cell cultures were normalized for comparing the results with other investigators who all used PBMC cultures with adjusted cell counts.

Table 1. Median values of the differential white blood cell counts and lymphocyte subsets in the peripheral blood of children and adults

		Children < 10 years		Children ≥ 10 years		Adults	
		Median	Q1 – Q3	Median	Q1 – Q3	Median	Q1 – Q3
White blood cell count (cells/l)	Leukocytes	6.1×10^9	$(5.0 - 7.1 \times 10^9)$	5.7×10^9	$(4.6 - 6.5 \times 10^9)$	4.6×10^9	$(4.0 - 5.7 \times 10^9)$
	Lymphocytes	3.1×10^9	$(2.1 - 3.7 \times 10^9)$	2.1×10^9	$(1.6 - 2.6 \times 10^9)$	1.5×10^9	$(1.2 - 2.0 \times 10^9)$
	Monocytes	3.3×10^8	$(2.9 - 5.3 \times 10^8)$	35×10^8	$(2.7 - 5.2 \times 10^8)$	2.5×10^8	$(1.8 - 3.9 \times 10^8)$
	PBMC	3.4×10^9	$(2.5 - 4.0 \times 10^9)$	2.7×10^9	$(1.9 - 3.1 \times 10^9)$	1.8×10^9	$(1.4 - 2.4 \times 10^9)$
Lymphocyte subsets (% of all lymphocytes)	CD3 ⁺ T cells	72	(63 – 78)	68	(64 – 73)	73	(67 – 78)
	CD19 ⁺ B cells	16	(13 – 19)	14	(10 – 16)	9	(6 – 12)
	CD16 ⁺ /CD56 ⁺ NK cells	13	(10 – 18)	16	(12 – 21)	17	(12 – 23)
	CD4 ⁺ cells	45	(38 – 49)	42	(34 – 45)	52	(42 – 56)
	CD8 ⁺ cells	30	(28 – 36)	34	(30 – 41)	35	(30 – 42)
	HLADR ⁺ T cells	8	(5 – 10)	7	(5 – 8)	8	(5 – 12)

IFN- γ production in the whole blood cell cultures of children and adults

In the blood cell cultures of the younger children (< 10 years), after PHA stimulation but not after PWM stimulation, lower levels of IFN- γ were measured ($p \leq 0.001$) whereas in the group of the older children (≥ 10 years) the values were above the younger children but below the adults (Table 2). There was no difference in the IFN- γ values between male and female subjects.

When the different absolute mononuclear cell counts were taken into account leukocytes of both groups of children produced significantly ($p \leq 0.001$) less IFN- γ than adult leukocytes after stimulation with PHA and PWM (Table 3). The individual values in Fig. 1 show a gradual elevation of the leukocyte IFN- γ production during childhood.

IL-1- α and IL-1- β production

In the supernatants of the PHA stimulated whole blood cell cultures of the group 1 and group 2 children IL-1- α and IL-1- β concentrations were measured that were comparable to those of the adults. After PWM stimulation IL-1- β levels were somewhat higher in both groups of children (Table 2).

After normalization of the absolute PBMC counts IL-1- α and IL-1- β production was significantly lower in the group of children under 10 years ($p \leq 0.001$ for IL-1- α and $p \leq 0.05$ for IL-1- β) but not in the group of the older children as compared to the adults (Table 3).

IL-2 production

IL-2 levels in the blood cell cultures of the children were comparable to the adults after stimulation with both mitogens. After normalization of the absolute PBMC counts the relative IL-2 production after stimulation with PHA and PWM was significantly lower in both groups of children ($p \leq 0.001$). Data are given in Table 2 and Table 3 respectively.

TNF- α production

In the whole blood cell cultures of both groups of children, significantly ($p \leq 0.01$) lower TNF- α levels were measured compared to the adults (Table 1). The same result was obtained after normalization of the absolute PBMC counts. The increasing values in both groups of children and the adults are shown in Fig. 2.

Table 2. Median values of the measured cytokine levels in stimulated whole blood cell cultures of healthy children at different ages and differences from adults (mitogen: PHA 10 mg/l and PWM 5 mg/l)

		Adults		Children aged ≥ 10 years		Children aged < 10 years	
		Median values (ng/l)		Median values (ng/l)	Difference from adults	Median values (ng/l)	Difference from adults
IFN- γ	PHA	48×10^3		31×10^3	n.s.	13×10^3	▼ $p \leq 0.001$
	PWM	61×10^3		54×10^3	n.s.	56×10^3	n.s.
IL-2	PHA	1800		1300	n.s.	1200	▼ $p \leq 0.05$
	PWM	1500		1400	n.s.	1300	n.s.
IL-1- α	PHA	90		100	n.s.	80	n.s.
	PVVM	250		270	n.s.	270	n.s.
IL-1- β	PHA	810		1100	n.s.	870	n.s.
	PVVM	2300		3100	▲ $p \leq 0.05$	2900	▲ $p \leq 0.01$
TNF- α	PHA	920		490	▼ $p \leq 0.01$	340	▼ $p \leq 0.001$
	PWM	510		300	▼ $p \leq 0.05$	390	n.s.

n.s. = not significant, ▲ = elevated, ▼ = decreased in comparison to adults.

Table 3. Median values of the relative leukocyte cytokine production in stimulated whole blood cell cultures of children at different ages and adults after normalization of the PBMC counts

	Mitogen	Adults	Children aged ≥ 10 years		Children aged < 10 years	
		Median values (ng/l)	Median values (ng/l)	Difference from adults	Median values (ng/l)	Difference from adults
IFN- γ	PHA	56×10^3	25×10^3	$\nabla p \leq 0.001$	11×10^3	$\nabla p \leq 0.001$
	PWM	74×10^3	51×10^3	$\nabla p \leq 0.01$	35×10^3	$\nabla p \leq 0.001$
IL-2	PHA	2300	910	$\nabla p \leq 0.001$	940	$\nabla p \leq 0.001$
	PWM	1800	1100	$\nabla p \leq 0.05$	960	$\nabla p \leq 0.001$
IL-1- α	PHA	100	90	n.s.	50	$\nabla p \leq 0.001$
	PWM	290	300	n.s.	200	$\nabla p \leq 0.001$
IL-1- β	PHA	960	820	n.s.	610	n.s.
	PWM	2700	3000	n.s.	2300	$\nabla p \leq 0.05$
TNF- α	PHA	1200	530	$\nabla p \leq 0.001$	230	$\nabla p \leq 0.001$
	PWM	680	390	$\nabla p \leq 0.01$	340	$\nabla p \leq 0.001$

n.s. = not significant, ∇ = decreased.

Discussion

Until now investigations of the developing immune system have been restricted to newborn and very young children. Therefore we extended our study to children and adolescents 1 to 17 years of age, com-

paring their leukocyte cytokine production with that of healthy adults. The relatively large number of 52 children enabled us to distinguish 2 statistically relevant groups with young children under 10 years and older children 10 years of age and older.

In the supernatants of the whole blood cell cultures of the children we measured lower levels of the cytokines; however, the differences from the adults were only significant for IFN- γ , IL-2, and TNF- α after PHA stimulation.

The results became clearer when the higher absolute lymphocyte and monocyte counts in the peripheral blood of the children were taken into account. After normalization of the absolute PBMC counts it could be shown that in the group of the children

IFN-gamma [μ g/ml]

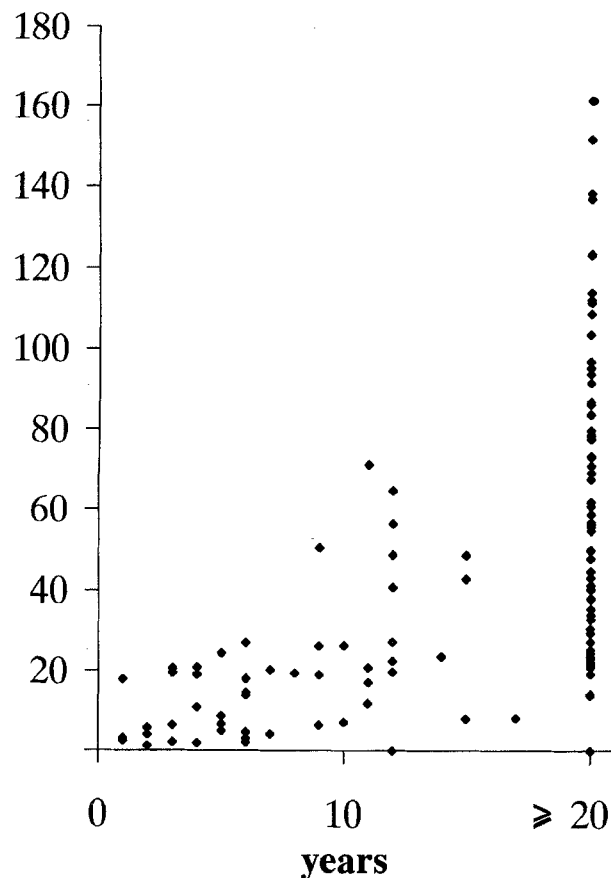


Fig. 1. Individual values of IFN- γ production of leukocytes from children at different ages and adult persons (mitogen: PWM 5 mg/l; incubation time: 4 days).

TNF- α [ng/l]

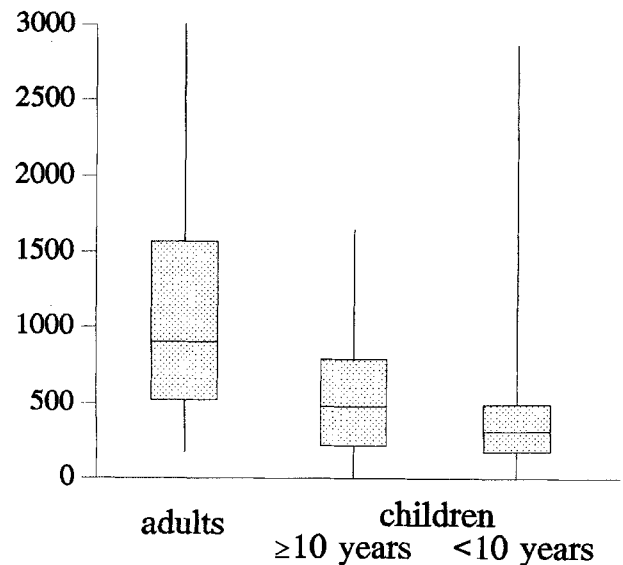


Fig. 2. Box plots of the TNF- α values in whole blood cell cultures from healthy children and adult persons (mitogen: PHA 10 mg/l; incubation time: 4 days).

aged below 10 years the leukocytes produced relatively lower amounts of all measured cytokines while in the group of the children 10 to 17 years of age the leukocytes produced relatively lower amounts of IL-2, IFN- γ , and TNF- α after stimulation with both mitogens. After this correction it is possible to compare our results with those of other authors, who all used cultures with isolated PBMC and adjusted cell counts. Our findings are in accordance with other publications where, however, to our knowledge, only newborn and very young children were studied. English et al. found, for instance, a lower production of TNF and lymphotoxin by human neonatal mononuclear cells after stimulation with PHA as compared to adult controls (3). Also, a decreased expression and production of IFN- γ by PHA stimulated cord blood leukocytes has been shown by several other authors (4–7), and this impaired IFN- γ response was considered to be either the consequence of a low macrophage activity or the consequence of an exaggerated cellular sensitivity to the suppressive effects of PGE produced endogenously in normal amounts (8). In the last years it has also been found that variations in the rate of postnatal maturation of T-cell competence as shown by the leukocyte IFN- γ and IL-4 production pattern may be associated with the development of atopy (9–13).

Our study showed a clear rise in the cytokine production of leukocytes from the younger to the older children and the adults. Regarding the measured cytokine levels as parameters for T-cell activity we thus could show in a relatively large group of children that the peripheral T-cell function at least in respect to lymphokine production is insufficient during childhood relative to adulthood which is partly compensated by a higher absolute lymphocyte and monocyte count in the peripheral blood of the children. However, there seems to be a gradual development of these immune functions during childhood and adolescence, a process that ends only in young adults.

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