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Immunoregulation of immunoglobulin production in normal infants and children

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SUMMARY

Proportion of T cell subsets, spontaneous and PWM stimulated immunoglobulin production by peripheral blood lymphocytes and concanavalin A- (Con A) stimulated suppressor cell activity on immunoglobulin production by B cells was studied in 37 infants and children, to investigate changes of these parameters with age. Proportion of suppressor/cytotoxic ($T8^+$) T lymphocytes was significantly lower in children below the age of 5 years, while there was no difference in proportion of total T lymphocytes ($T3^+$) and helper/inducer ($T4^+$) T cells. Spontaneous production and secretion of IgG and IgM by lymphocytes from children of all age groups was similar to that found in adults, but lymphocytes of children below the age of 10 years showed a low response to PWM stimulation. The activity of Con A-induced suppressor cells in inhibiting B cells producing immunoglobulins was almost absent in infancy, gradually increased during childhood and reached adult levels in teenagers. A significant association between the proportion of $T8^+$ cells and Con A-induced suppression of B cell proliferation and a relationship between $T4^+$ cells and spontaneous Ig production indicated the increasing maturity with respect to both number and function of peripheral blood lymphocyte subsets with advancing age.

Keywords Con A-induced suppression immunoglobulin production immunoregulation children

INTRODUCTION

Studies performed on cord blood have shown that several immune functions at birth differ profoundly from those in adult life. Newborns' lymphocytes produce less IgG and IgA (Andersson *et al.*, 1981), contain a lower proportion of suppressor/cytotoxic T cells (Hayward & Merrill, 1981), but show an increase in T suppressor cell activity (Jacoby & Oldstone, 1983). Very little is known about the changes of these various immune functions with age. We have therefore investigated *in vitro* production of IgM and IgG, effect of pokeweed mitogen (PWM) stimulation on immunoglobulin production, proportion of functionally distinct T lymphocyte subsets and function of suppressor T cells in children from infancy to adolescence.

MATERIALS AND METHODS

Subjects. Thirty-seven normal healthy infants and children were studied, after informed consent

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was obtained from their parents: 19 subjects were aged between 4 weeks and 5 years (median age 1 year 3 months) nine between 5 years and 10 years (median age 7 years 9 months), and nine between 10 years and 16 years (median age 12 years 6 months). There were 20 females and 17 males. In addition, 27 healthy adult volunteer laboratory personnel, aged between 20 and 40 years were studied.

Cell preparation. Peripheral blood was mixed with 6% dextran (wt/vol.) in 0·15 M sodium chloride and preservative free heparin (10 u/ml blood) and the red cells allowed to settle at 37°C for 45 min. The leucocyte rich plasma was layered on Ficoll-Triosil density gradients and centrifuged for 20 min at 400 ***g*** (Böyum, 1968). Mononuclear cells were harvested from the gradient interface and then incubated in plastic Petri dishes for 45 min at 37°C, to deplete adherent cells; the resulting preparation contained 3–5% monocytes identified by peroxidase staining (Preud'Homme & Flandrin, 1974). The viability according to trypan blue exclusion was greater than 95 %. The lymphocytes were washed three times with Hank's balanced salt solution (HBSS) (Wellcome) and were resuspended at a concentration of 1×10^6 cells/ml in RPMI 1640 (GIBCO) supplemented with glutamine (2 mM), penicillin (200 u/ml), streptomycin (100 μg/ml), amphotericin B (2μg/ml) and 20% heat-inactivated fetal calf serum (GIBCO).

Culture conditions. Peripheral blood lymphocytes (PBL) were cultured at a concentration of 2×10^5 cells per well in flat bottomed microplates (Sterilin). PWM (Sigma Ltd) was added at a concentration of 20 μg/ml, and plates incubated for 7 days at 37°C in humidified air containing 5% CO_2, 12% O_2 and 83% N_2.

To stimulate suppressor T cells (Shou, Schwartz & Good, 1976), 5×10^6 lymphocytes were incubated with 20 μg/ml of concanavalin A (Con A) (Sigma Chemical Co.) at 37°C for 24 h, under conditions described above. Following incubation, the Con A treated lymphocytes were washed twice with HBSS containing 0·3 M α-methyl-D-mannoside (Sigma), to remove Con A and then once in HBSS and reconstituted to 1×10^6 cells/ml in supplemented RPMI 1640. Equal numbers of Con A stimulated and unstimulated lymphocytes were co-cultured in the presence of 20 μg/ml of PWM and incubated at 37°C for 6 days as already described.

Evaluation of immunoglobulin production. Plaque assay. After 7 days of culture, the cells were washed three times in RPMI 1640 and resuspended at a concentration of 1×10^5 cells/ml. A 100 μl aliquot of cells were mixed with 50 μl of sheep red cells coated with Staphylococcal protein A (Pharmacia), using chromium chloride (Gold & Fudenberg, 1967), 50 μl of an appropriately diluted IgG fraction of rabbit anti-human IgG or IgM (Miles) and 700 μl of agarose (Indubiose A37) at 46°C, poured into plastic Petri dishes and incubated at 37°C for 4 h. Guinea pig complement (1 ml) (Flow Laboratories) diluted 1:25 in RPMI 1640 was then added to each plate, and incubated for a further 2 h at 37°C. Discrete concentric areas of haemolysis were counted in indirect light and the results expressed as the number of immunoglobulin producing cells per million viable lymphocytes (Hammarström *et al.*, 1979).

Immunofluorometric assay. Supernatants from the microplate wells were removed at the end of the culture period and stored at −20°C until required for analysis. Following thawing and centrifugation at 1,700***g*** for 10 min to remove cell debris, IgM and IgG were determined using an immunofluorescent assay kit (Immuno-Fluor, Bio-Rad, Richmond, California). The supernatants were incubated for 90 min at 37°C with beads coated with anti-IgM or anti-IgG. The appropriate fluorescein labelled immunoglobulin reagent was then added and following incubation for a further 60 min, the beads were washed three times in buffer and finally resuspended in 2·5 ml buffer. Reference IgM and IgG standards were included in each assay and the fluorescence of each sample read in a spectrofluorometer (Perkin–Elmer) at excitation and emission wavelengths of 485 nm and 525 nm, respectively. The results are expressed as the amount of IgG or IgM per 10^6 lymphocytes (Schwartz, 1980).

The degree of suppression was calculated according to the following formula:

$$\% \text{ suppression} = \left[1 - \frac{\text{No. of plaque-forming cells or amount of immunoglobulin produced by PWM-stimulated cells + Con A}}{\text{No. of plaque-forming cells or amount of immunoglobulin produced by PWM-stimulated cells}}\right] \times 100$$

Enumeration of T cell subsets. Commercially available murine monoclonal antibodies were used (Ortho Pharmaceutical Corporation). It has been claimed that T3 identifies total T cell populations, T4 identifies helper/inducer T lymphocytes and T8 identifies suppressor/cytotoxic cells. PBL were

incubated with each of these antibodies for 30 min at 4°C, washed twice with HBSS and then incubated under similar conditions with fluorescein labelled goat anti-mouse immunoglobulin diluted 1:50. After three further washes, the cells were mounted and counted under a Leitz Orthoplan u.v. microscope, the number of positive cells being expressed as a percentage of total lymphocytes counted (Reinherz *et al.*, 1980).

Statistics. Statistical analysis was performed using the Wilcoxon rank sum test and the correlation coefficient for linear regression.

RESULTS

In some of the children it was impossible to perform all the planned experiments due to the low number of lymphocytes obtained. This was particularly so in very young children. The number of children in whom the single experiments have been performed is reported in each table.

IgM production

The median number of lymphocytes spontaneously producing IgM as identified by the haemolytic plaque assay, at the end of the 7 day culture period, was similar for all the age groups studied (Table 1). Following stimulation with PWM, there was an increase in the number of IgM producing cells, the stimulation index increasing with advancing age. However, the number of plaque forming cells after PWM stimulation was significantly lower in all the children when compared to adults ($P < 0{\cdot}01$). The results of IgM secretion into the culture media as measured by the fluorometric assay were similar. PWM stimulation increased IgM secretion but less IgM was secreted in all the children when compared to adults ($P < 0{\cdot}01$).

Con A-induced suppression of IgM synthesis. Con A-activated suppression of IgM synthesis tended to increase with age but did not differ significantly for any of the groups studied (Table 2). The inhibition of IgM secretion by Con A-activated suppressor cells followed a similar pattern, showing a tendency to increase with increasing age.

IgG production

The median number of lymphocytes spontaneously producing IgG at the end of the culture period was similar for all age groups including adults (Table 3). Following stimulation with PWM, the number of lymphocytes producing IgG increased in all the groups, but less in children than in adults ($P < 0{\cdot}01$). The spontaneous secretion of IgG into the culture medium over the 7 day culture period was similar for all groups studied. In children below the age of 10 years there was no response to PWM stimulation, whereas in children above the age of 10 years PWM increased IgG secretion.

Con A-induced suppression of IgG synthesis. Con A-activated suppression of proliferation of IgG producing B cells was significantly less in all the children than in adults ($P < 0{\cdot}01$) (Table 4). Such suppression gradually increased with age but was still significantly lower in teenagers. The inhibition of immunoglobulin secretion follows a similar pattern to that described for proliferation, being low in infants and increasing with age. Suppression of IgG secretion was significantly lower in children below the age of 10 years when compared to adults ($P < 0{\cdot}05$).

There was a significant correlation between the increase in Con A-induced suppression of PWM-induced IgG production as measured by the haemolytic plaque assay and increasing age ($r = 0{\cdot}73$, $P < 0{\cdot}001$) (Fig. 1).

Monoclonal antibody staining of lymphocytes

The relative proportion of $T3^+$ cells (total peripheral T cells) and $T4^+$ cells (helper/inducer) showed no significant differences between the different age groups. In contrast the proportion of $T8^+$ cells (suppressor/cytotoxic) was significantly lower ($P < 0{\cdot}02$) in children under the age of 5 years than in adults (Table 5). The ratio of helper to suppressor cells ($T4^+/T8^+$) was significantly higher ($P < 0{\cdot}01$) in children below the age of 5 years but was not significantly different from adults in children above the age of 5 years. Sufficient numbers of lymphocytes were obtained from 18 of 27 children to compare number and function of suppressor cells. Suppressor cell activity (as assessed

Table 1. Spontaneous and PWM-induced IgM production in normal infants, children and adults controls

Age	Number of IgM PFC/10^6 cells		Stimulation index	Amount of IgM secreted ng/10^6 cells		Stimulation index
	Spontaneous	PWM		Spontaneous	PWM	
1 month–< 5 years	113 (51–357) $n=9$	202 (100–1,588) $n=13$	1·78 (1·1–2·7)	217 (75–1,025) $n=9$	335 (160–1,025) $n=12$	1·54 (0·8–3·5)
5 years–< 10 years	84 (53–1,395) $n=7$	156 (92–2,461) $n=7$	1·85 (1·2–3·4)	283 (160–440) $n=7$	514 (160–1,350) $n=7$	1·81 (0·61–3·5)
10 years–16 years	84 (159–1,185) $n=9$	352 (89–5,416) $n=9$	4·19 (0·9–9·4)	225 (75–400) $n=6$	489 (160–1,820) $n=7$	2·17 (1·6–5·2)
20 years–40 years	92 (20–835) $n=27$	1656 (192–15,692) $n=27$	18 (4·8–32)	414 (100–1,375) $n=19$	1034 (350–4,500) $n=21$	2·5 (1·8–3·8)

Data expressed as median and range.
PFC = plaque forming cells.
PWM = pokeweed mitogen.

Table 2. Con A-induced suppression of PWM-stimulated IgM synthesis in normal infants, children and adult controls (mean ± s.d.)

Age (median age)	Number	Percentage suppression of proliferation	Number	Percentage suppression of secretion
1 month–< 5 years (1 year 6 months)	13	37·2 ± 45	12	37·7 ± 24·4
5 years–< 10 years (8 years)	7	49·0 ± 51	7	41·4 ± 34
10 years–16 years (12 years 6 months)	9	70·5 ± 40	7	50·4 ± 25·6
20 years–40 years (31 years)	27	89·4 ± 10	21	70·1 ± 16·5

Table 3. Spontaneous and PWM-induced IgG production in normal infants, children, and adults controls

	Number of IgG PFC/10^6 cells			Amount of IgG secreted ng/10^6 cells		
Age	Spontaneous	PWM	Stimulation index	Spontaneous	PWM	Stimulation index
1 month–< 5 years	448 (111–1,357) $n=13$	723 (241–1,750) $n=18$	1·61 (0·9–1·9)	337 (100–1,125) $n=13$	302 (100–825) $n=16$	0·89 (0·5–2·4)
5 years–< 10 years	485 (160–1,077) $n=9$	500 (149–1,384) $n=9$	1·03 (0·5–1·9)	588 (150–2,540) $n=9$	616 (200–2,075) $n=9$	0·90 (0·3–2·0)
10 years–16 years	537 (159–1,428) $n=9$	681 (192–4,250) $n=9$	1·26 (0·7–3·8)	425 (125–725) $n=9$	662 (200–3,900) $n=9$	1·55 (1·0–5·1)
20 years–40 years	455 (62–2,152) $n=22$	2182 (450–16,875) $n=27$	4·79 (2·4–8·4)	370 (100–1,850) $n=22$	787 (225–2,600) $n=25$	2·12 (1·9–7·4)

Data expressed as median and range.
PFC = plaque forming cells.
PWM = pokeweed mitogen.

Table 4. Con A-induced supression of PWM-stimulated IgG production in normal infants, children and adult controls (mean ± s.d.)

Age (median age)	Number	Percentage suppression of proliferation	Number	Percentage suppression of secretion
1 month–< 5 years (1 year 3 months)	19	21·8 ± 25·8*	18	20·1 ± 20·0**
5 years–< 10 years (7 years 9 months)	9	56·5 ± 15·3*	9	32·0 ± 19·0**
10 years–16 years (12 years 6 months)	9	61·7 ± 10·8*	9	46·2 ± 18·5
20 years–40 years (31 years)	25	82·0 ± 10·7	25	63·0 ± 17·6

* $P < 0·01$.
** $P < 0·05$.

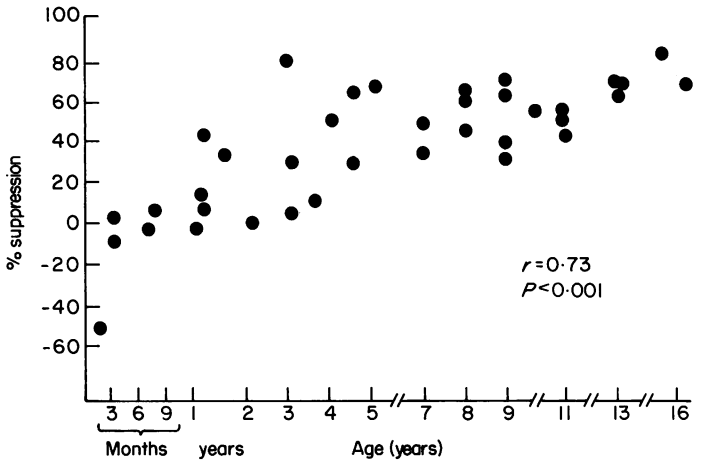

Fig. 1. Relationship between Con A-induced suppression of PWM induced IgG production and age.

Table 5. Proportion of T cell subsets and ratio of $T4^+/T8^+$ cells in normal infants, children and adult controls (mean ± s.d.)

Age (median age)	Number	Proportion of positive T cells			Ratio T4/T8
		T3	T4	T8	
1 month–< 5 years (1 year 6 months)	9	65·3 ± 8·7	48·3 ± 4·9	19·7 ± 6·3*	2·68 ± 0·78
5 years–< 10 years (8 years)	8	65·7 ± 10·1	44·2 ± 7·8	24·1 ± 7·6	2·10 ± 1·14
10 years–16 years (12 years 6 months)	9	60·0 ± 8·3	43·5 ± 5·6	22·8 ± 5·2	1·99 ± 0·69
20 years–40 years (31 years)	18	63·0 ± 7·5	41·1 ± 7·2	28·0 ± 4·1	1·5 ± 0·4

* $P < 0{\cdot}02$

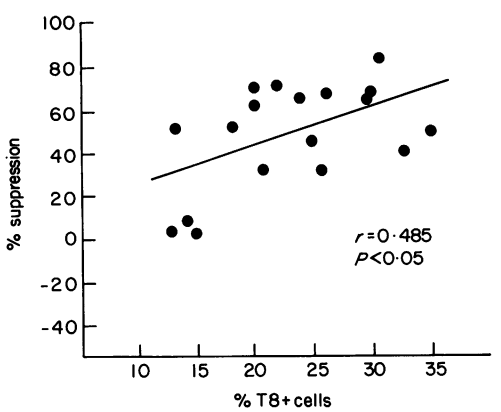

Fig. 2. Relationship between Con A-induced suppressor cell function and proportion of $T8^+$ cells.

by the haemolytic plaque assay) correlated weakly with the number of $T8^+$ cells ($r = 0{\cdot}485$, $P < 0{\cdot}05$) (Fig. 2), but no correlation was found between the number of helper cells and suppressor cell activity. However, there was a significant correlation between the number of $T4^+$ cells and the amount of spontaneously secreted IgM ($r = +0{\cdot}58$, $P < 0{\cdot}05$). There was a trend for the amount of spontaneous IgG secretion to correlate with the proportion of helper cells but this did not reach statistical significance ($r = 0{\cdot}40$, $P =$ ns). There was no correlation between the number of suppressor cells and spontaneous IgM and IgG secretion, nor was there a correlation between the number of helper cells and the amount of IgM and IgG secreted into the culture medium following PWM stimulation.

DISCUSSION

Our results show that spontaneous production and secretion of IgG and IgM by *in vitro*-cultured lymphocytes from children of all age groups are similar to those observed in adults. The effect of PWM stimulation, however, is different from adults, since lymphocytes from the children produced and secreted significantly smaller quantities of IgG and IgM in response to this mitogen. This was particularly evident in children below the age of 10 years. Such a poor response to PWM has been previously observed on cord blood (Andersson *et al.*, 1983) and has been attributed to the absence of T helper activity in the newborn. An alternative explanation, however, has been proposed by Durandy, Fischer & Griscelli (1979) and Tosato *et al.* (1980) who showed that cord blood lymphocytes can synthesize immunoglobulins in response to Epstein–Barr (EB) virus but not to PWM and that in co-culture experiments, cord blood T cells inhibit immunoglobulin production by adult cells stimulated with PWM but not with EB virus, suggesting that cord blood contains a population of suppressor cells that are activated by PWM. Persistence of these PWM sensitive suppressor cells after birth and their gradual disappearance with age could be the cause of the observed poor response to PWM in very young children and of its improvement with age.

Children below the age of 5 years also have a low proportion of $T8^+$ cells. Although similarly low numbers have been described in neonates (Hayward & Merrill, 1981), this was not accompanied by impaired suppressor function. On the contrary, neonates showed high suppressor-cell activity, possibly exerted by $T8^-$ cells. In the present study, low proportion of $T8^+$ cells in very young children parallels low Con A stimulated suppressor cell regulation of IgG production, with both parameters increasing steadily throughout childhood, in agreement with the results of Miyawaki *et al.* (1981) who used a comparable assay. Although these findings may reflect low responsiveness of suppressor cells to Con A in early childhood, it is also possible that they are artefactual. Con A-induced suppression is measured as inhibition of PWM-induced immunoglobulin production in an assay established in normal adults who respond to PWM stimulation by producing immunoglobulins. If PWM sensitive T suppressor cells are present in young children, it is possible that addition of Con A activated cells cannot further affect immunoglobulin production.

Correlations between the proportion of $T8^+$ cells and suppressor cell activity and between the proportion of $T4^+$ cells and spontaneous immunoglobulin secretion, demonstrated in the present study, suggest that the relative lack of immunoregulatory control of B cell function in infancy is probably a reflection of the low numbers of mature T cells. The numbers of such cells increased gradually throughout childhood and this is accompanied by increasing suppressor function.

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