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Lymphocyte subsets in term and significantly preterm UK infants in the first year of life analysed by single platform flow cytometry

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Summary

This observational study describes the ranges observed for lymphocyte subsets for significantly preterm infants (<32 weeks) in the first year of life, measured by single platform flow cytometry and compared to identically determined subsets in term infants. After ethical approval 39 term and 28 preterm infants had lymphocyte subset analysis before and after their primary immunization series. Median values with 5th and 95th percentiles of absolute counts and percentages are presented for total lymphocytes, T cells, NK cells, B cells, cytotoxic T cells, helper T cells, dual positive T cells, activated T cells, activated T helper cells (including T regulatory cells), pan memory T cells, pan naïve T cells, memory helper T cells, naïve helper T cells and the T helper/suppressor ratio. The lymphocyte profile of the preterm infants differed from that of the term infants.

Keywords: Lymphocytes, flow cytometry, infant, preterm

Introduction

The lymphocytic phenotype of an individual infant can now be readily measured by single platform flow cytometry generating both absolute and proportional counts. These systems utilize tubes that contain a known number of brightly fluorescent polystyrene beads to which a known volume of blood is added. The absolute count is then calculated from the ratio of beads to cells in the tube. This has been recognized by the United Kingdom National External Quality Assurance Scheme (UK NEQAS) as the most appropriate and accurate method for lymphocyte subset analysis [1]. Without appropriately generated normal ranges interpretation of results in clinical situations is difficult, and may lead to misdiagnosis or inappropriate treatment, yet data on lymphocyte subsets in infants (term or preterm) generated by single platform technology does not exist.

Existing normal range data on infant lymphocyte subsets generated by techniques other than single platform flow cytometry may be inaccurate because of a number of problems: erythroid cell precursor contamination in the neonate [2], the use of mononuclear cell separation rather than whole blood lysis (which better preserves the original white blood cell distribution [3]), a lack of consideration of absolute as well as relative counts [4], and the loss of preservation of subsets present in small numbers that occurs with dual platform techniques [1]. It also has the benefit of requiring only a single small volume sample, helpful when studying small preterm neonates. Both studies that previously analysed reasonable numbers of infants (less than one year of age) used dual platform techniques [5,6], and did not include a preterm population, existing studies of which are small and of limited applicability for similar reasons [7,8].

As part of a study of immunization responses in preterm neonates we analysed lymphocyte subsets in infants' before and on completion of standard UK primary immunization (given at 2, 3 and 4 months of age). We present data in term and preterm infants generated by single platform analysis with the TruCount system (Becton-Dickinson, San Jose, USA).

Methods

Patients

Term infants (>37 completed weeks of gestation) were recruited from a single site in Northern England between March and May 2002; preterm infants (<32 completed weeks of gestation) were recruited from the four tertiary neonatal units in the former Northern Health Region of England between February 2001 and July 2002. Ethical approval was obtained from each Local Research Ethics Committee and

Table 1. Antibodies used during analysis.

Antibody	Clone	Format
CD3/CD8/CD45/CD4	SK7, SK1, 2D1, SK3	FITC, PE, PerCP, APC
CD3/CD16+ CD56/CD45/CD19	SK7, B73·1, NCAM16·2, 2D1, SJ25C1	FITC, PE, PerCP, APC
CD3	SK7	FITC, PERCP
CD4	SK3	APC
CD25	2A3	PE
CD45	2D1	PerCP
CD45RA	L48	FITC
CD45RO	UCHL-1	PE
Anti-HLA-DR	L243	PE

Antibodies from BD Sciences.

written consent obtained from all parents. Sufficient blood was available for lymphocyte analysis in 39 term infants and 28 preterm infants. Demographic details were obtained from patient records.

Laboratory methods

Peripheral blood was taken just before and 6–8 weeks after completion of primary series immunization for measurement of lymphocyte subsets. Topical anaesthetic cream (Ametop™ Smith and Nephew) was applied. Whole blood was taken into EDTA for lymphocyte analysis. Fifty microlitres of well mixed whole blood and 20 microlitres of monoclonal antibody (Table 1) were added to the TruCount tubes, vortexed and incubated for 15 min at room temperature, 450 µl of lysing reagent was added, vortexed and incubated for a further 15 min at room temperature (FACS lysing solution, Becton Dickinson). Samples were analysed on a FACSCalibur four colour dual laser bench top flow cytometer using MultiSET software (Becton Dickinson, San Jose, CA, USA) in the regional immunology laboratory at the Royal Victoria Infirmary, Newcastle. The gating strategy used was CD45 *versus* side scatter.

Statistical analysis

Data were skewed, and are therefore presented as medians with 5th and 95th centiles. The term and preterm populations were compared by standard nonparametric tests (Mann–Whitney *U*-test). Analyses were performed in SPSS version 11·0.

Results

Summary data for demography and sample timing are presented in Table 2. The lymphocyte subset data are presented in Tables 3, 4 and 5.

Statistically significant differences were demonstrated between term and preterm populations in several subsets. Preterm infants had lower counts of absolute lymphocytes, T cells, B cells, and T helper cells than term infants when initially tested (7–8 weeks of age). The CD4/CD8 ratio was lower in preterm infants at this point. In addition within the T cell subset a larger proportion of helper T cells expressed CD25 and a smaller proportion of all T cells expressed the naïve (CD45RA) phenotype.

By the second analysis, at around 7 months of postnatal age, the B cell numbers in the preterm group were term equivalent, but the reduced absolute lymphocyte count, total T cell count, and T helper count were persistent, as was the reduced proportion of pan naïve T cells.

Discussion

We have presented data on ranges of lymphocyte subpopulations in term and significantly preterm infants analysed

Table 2. Study population and timing of samples.

	Preterm ($n = 28$)		Term ($n = 39$)	
Median (IQR)	Pre-immunization ($n = 18$)	Post immunization ($n = 24$)	Pre-immunization ($n = 36$)	Post immunization ($n = 29$)
Birthweight (g)	905 (718–1100)	995 (738–1380)	3652 (3167–3871)	3630 (3188–3870)
Gestation (weeks)	26·5 (24·6–28·7)	28 (25·2–30)	39·8 (39–41·1)	40·4 (39·6–41·0)
Male (*n* (%))	12 (67)	15 (63)	16 (44)	15 (51)
Multiple birth (*n* (%))	6 (33)	6 (25)	3 (8)	2 (7)
Postnatal steroids (*n* (%))	3 (17)	4 (17)	0	0
Days ventilated	6 (1–25)	3·5 (1–23)	0	0
Postnatal age analysed	8·1 (6·6–8·6) (weeks)	6·9 (6·4–7·6) (months)	7 (6·4–7·4) (weeks)	6·3 (6·0–6·6) (months)

Table 3. Peripheral blood lymphocyte subsets identified.

Name	CD marker	Subgroup for proportions
Total lymphocyte count	CD45	–
T cell	CD3	CD45
Natural killer cell	CD16CD56CD3-	CD45
B cell	CD19	CD45
T suppressor cell	CD3CD8	CD45
T helper cell	CD3CD4	CD45
Dual positive T cell	CD3CD8CD4	CD45
Activated T cell (including T regulatory cells)	CD3HLADR	CD3
Activated T helper cell	CD3CD4CD25	CD3
Pan memory T cell	CD3CD45RO	CD3
Pan naïve T cell	CD3CD45RA	CD3
Memory helper T cell	CD3CD4CD45RO	Absolute count only
Naïve helper T cell	CD45RA-CD3CD4CD45RA	Absolute count only
T helper/suppressor ratio	CD45RO-CD4/CD8	–

with a single platform technique as recommended by UK NEQAS. The data for term infants are numerically in keeping with that of the most suitable comparative data previously published, although both studies that previously analysed reasonable numbers of infants (less than one year of age) used dual platform techniques [5,6].

No attempt has been made within the preterm population to account for factors that have previously been thought to affect the peripheral blood lymphocyte phenotype, such as mode of delivery [9], antenatal steroid use [8,10,11], and maternal pre-eclampsia [12,13]. Such factors are inevitable within a 'typical' population of significantly preterm infants, hence 'correcting' for them when describing normal ranges seems inappropriate. We believe that both the term and preterm cohorts described are representative of their larger populations and therefore the data are descriptive of the normal ranges expected within these populations. This data will aid immunology laboratories analysing samples from term and preterm infants in the first year of life to help clinicians in practice determine whether individual infants merit further investigation: those with subsets outside the 5th and 95th centiles probably do.

The differences between term and preterm infants are interesting. There is no good comparative data; other studies use different methodology, or assess infants immediately at birth (via cord blood). The differences observed may represent on-going development of the immune repertoire, or exhaustion of the neonatal pool of lymphocytes in association with preterm birth and its attendant stresses in a manner akin to that documented for neonatal neutrophils. In view of the increased propensity of the preterm neonate to infection, as well as the increasing evidence of reduced responses to immunization in the preterm neonate, these differences merit further study.

Table 4. Pre-immunization lymphocytes subsets, absolute counts and proportions.

Subset	Preterm minus steroid recipients $n = (15)$	All preterm ($n = 18$)	Term	Steroid population	
				P^* if excluded	P^* if included
Total lymphocyte count	**4256 (2933–6245)**	**4180 (2411–6245)**	**5902 (3882–9184)**	**<0·001**	**<0·001**
%T cells	66 (45–77)	65 (45–77)	69 (59–78)	NS	NS
Absolute T cells	**2879 (1898–3878)**	**2816 (1519–3878)**	**4098 (2409–6693)**	**<0·001**	**<0·001**
% NK cells	7 (2–13)	7 (2–15)	5 (3–12)	NS	0·018
Absolute NK cells	283 (91–861)	314·5 (91–861)	277 (157–888)	NS	NS
% B cells	23 (18–47)	23 (18–47)	24 (16–35)	NS	NS
Absolute B cells	**1010 (710–2327)**	**931 (466–2327)**	**1481 (776–2358)**	**0·05**	**0·019**
%T suppressor cells	19 (13–32)	19 (13–34)	17 (8–27)	NS	NS
Absolute T suppressor cells	873 (454–1387)	810 (454–1855)	971 (509–1740)	NS	NS
%T helper cells	**46 (28–59)**	**45 (24–59)**	**50 (39–63)**	**0·07 (NS)**	**0·022**
Absolute T helper cells	**2210 (1090–2990)**	**1804 (1090–2990)**	**2946 (1659–5068)**	**<0·001**	**<0·001**
% Activated T helper cells	1 (0–2)	1 (0–2)	1 (0–3)	NS	NS
Absolute activated T helper cells	58 (9–114)	32·5 (8–114)	39 (14–164)	NS	NS
T helper/supresor ratio	**2·4 (1·1–4·1)**	**2·4 (0·69–4·1)**	**2·9 (1·5–6 7)**	**0·131 (NS)**	**0·04**
% activated T cells	3 (2–7)	3 (2–44)	4 (2–8·6)	NS	NS
% activated helper T cells	**12 (8–19)**	**12 (7–19)**	**9 (6–15)**	**<0·001**	**0·001**
% Pan memory T cells	37·5 (11–51)	37·5 (11–52)	33 (11–61)	NS	NS
% Pan naïve T cells	**87·5 (77–96)**	**85·5 (74–96)**	**92 (88–97)**	**0·01**	**0·003**
Absolute Memory helper T cells	1588 (913–1621)	1588 (913–1621)	1930 (1108–2752)	NS	NS
AbsoluteNaive helper T cells	378 (122–461)	378 (122–461)	–	–	–

Table shows median (5th–95th percentiles), % percentage of subgroup identified in Table 1, absolute count, cells/μl; NS = $P > 0·05$, * term *versus* preterm. Bold font indicates significant differences term *versus* preterm.

Table 5. Post immunization lymphocytes subsets, absolute counts and proportions.

Subset	Preterm minus steroid recipients $n = (20)$	All preterm ($n = 24$)	Term	Steroid population	
				P^* if included	P^* if excluded
Total lymphocyte count	**5892 (3193–9324)**	**5676 (3223–9224)**	**6555 (4747–10620)**	**0·032**	**0·08**
%T cells	64 (25–76)	64 (31·5–75·75)	67 (56·5–77·5)	NS	NS
Absolute T cells	**3847 (838–7123)**	**3606 (1115–6815)**	**4604 (2791–7939)**	**0·009**	**0·03**
% NK cells	**6 (2–8·9)**	**6 (2–10·5)**	**4 (3–10·5)**	**0·001**	**0·001**
Absolute NK cells	321·5 (183–715)	321·5 (183–694)	274 (134–794)	NS	NS
% B cells	28·5 (19–64)	28·5 (19–59)	28 (17–37·5)	NS	NS
Absolute B cells	1785 (825–2739)	1785 (816–2749)	1850 (1002–3360)	NS	NS
%T suppressor cells	16 (12–27)	16 (12–31)	17 (8·5–29·5)	NS	NS
Absolute T suppressor cells	997 (367–1984)	997 (399–1953)	1131 (548–2438)	NS	NS
%T helper cells	45 (14–59)	44·5 (18·2–59)	47 (35–58·5)	NS	NS
Absolute T helper cells	**2504 (422–5668)**	**2401 (643–5290)**	**3209 (1959–5983)**	**0·003**	**0·01**
% Activated T helper cells	1 (0–1·95)	1·00 (0·0–1·75)	1 (0–3·0)	NS	NS
Absolute activated T helper cells	35 (11·4–117)	32 (11·7–107)	40 (14·5–170)	NS	NS
T helper/supresor ratio	2·78 (1–4·4)	2·72 (1·0–4·3)	2·92 (1·3–6·5)	NS	NS
% activated T cells	4 (3–14)	4 (3–13·2)	5 (2·5–9·5)	NS	NS
% activated helper T cells	10 (6–14·9)	9·5 (6–14·7)	9 (6–14·5)	NS	NS
% Pan memory T cells	23 (11–71)	23 (11·2–65·2)	26 (13·7–58·7)	NS	NS
% Pan naïve T cells	**93 (87–99)**	**92 (87·4–98·6)**	**94·5 (88–100)**	**0·04**	**0·08**
Absolute Memory helper T cells	1964 (189–3132)	1749 (262–3115)	2403 (870–3890)	NS	NS
Absolute Naive helper T cells	166 (0–394)	173 (5·3–385·8)	163 (15·3–432·5)	NS	NS

Table shows median (5th–95th percentiles); %, percentage of subgroup identified in Table 1; absolute count, cells/μl; NS = $P > 0·05$; * term *versus* preterm. Bold font indicates significant differences term *versus* preterm.

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