

Identification of lymphocyte subsets in the newborn using a variety of monoclonal antibodies

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SUMMARY Using a small sample of peripheral venous blood, the normal range for lymphocyte subpopulations (T-cell, B-cell, T-suppressor cell, and T-helper cell) was defined in non-infected preterm and term infants. Lymphocyte subsets were identified using a variety of monoclonal antisera, and analysis was performed using a fluorescent activated cell sorter. Such methods allow an objective assessment of absolute numbers of cells per unit volume of whole blood. There was no significant difference in absolute numbers of lymphocyte subsets between term and preterm appropriate for gestational age (AGA) infants. Infants who were small for gestational age (SGA) had a significant deficiency in absolute numbers of total T-cells, helper and inducer T-lymphocytes, and B-cells compared with both term and preterm AGA infants. All newborn infants (term and preterm; AGA and SGA) had a highly significant increase in absolute numbers of both helper and suppressor T-lymphocytes compared with normal adults.

It is well known that the total lymphocyte count is increased in the newborn infant compared with adults.¹ The percentage of T-lymphocytes in the newborn defined by rosetting with sheep erythrocytes has been variously reported as normal or decreased in healthy term infants, although the absolute values are comparable with, or higher than adult values.² Several authors have shown that both the percentage and absolute numbers of B-lymphocytes are increased in neonates.³ Other studies indicate that the percentage of B-cells is lower in neonates, but agree that the absolute numbers are higher taking into account the increased total lymphocyte count.^{2,4} One study compared preterm (5 infants, gestational age range 28 to 36 weeks) with term infants, but could show no significant difference in the numbers of T- or B-cells.³ Nine preterm infants were studied by Hallberg and Hallberg,⁵ and 4 showed a decreased percentage of E-rosette forming cells in the first few days of life compared with adult controls. By age 3 weeks however, the percentage in all infants had reached adult levels.

The method of separation of human lymphocytes from erythrocytes and granulocytes can considerably bias the proportion of T- and B-lymphocytes and may account for the apparent differences obtained by others. We have found ficoll-hypaque gradient centrifugation to be unreliable, particularly in

neonates, with granulocytes frequently remaining at the ficoll-hypaque interface, and have therefore removed red cells by hydroxyethyl starch sedimentation and analysed all blood leucocytes. Our values are then expressed in terms of absolute numbers per unit volume of whole blood.

Materials and methods

Patients and samples. Venous blood samples (0.25-0.5 ml) were obtained from normal newborn infants (22 girls and 42 boys) who were undergoing venepuncture for routine clinical tests in the first week of life. All investigations were performed with the approval of the local ethical committee. Gestational age was assessed from the date of the last menstrual period and was confirmed in most cases by ultrasound examination in early pregnancy. The range was from 23 to 42 weeks. Mean birthweight was 2073 g (range 610-4880). Thirty-four infants were preterm (gestation 36 weeks or less) and their mean birthweight was 1708 g (range 610 to 2680). Ten infants (gestation range 27 to 40 weeks) were small for gestational age (SGA) and were below the 10th centile for weight. Their mean birthweight was 1654 g (range 680-2430). There was no evidence of intrauterine infection or congenital abnormalities in the SGA infants. Infants in whom infection was

strongly suspected or proved were excluded and none had been given a blood transfusion or exchange transfusion within the previous 48 hours.

Sixteen samples were taken within 24 hours of birth and the remaining 48 were obtained within the first week of life. Four infants were then followed with repeated analyses at weekly intervals for periods ranging from 4 to 9 weeks after birth. The normal adult control samples were obtained from healthy laboratory staff, and leucocytes were separated using the same technique as for the infant samples.

Leucocyte separation. Between 0.25 and 0.5 ml venous blood was collected in an heparinised tube and the leucocytes were separated within a few hours of venepuncture. The sample was diluted to 1 ml with tissue culture medium (TCM) (RPMI 1640: Gibco, Europe) and added to an equal volume of hydroxyethyl starch (Plasmasteril: Fresenius Bad, Hamburg) in a conical tube. After standing for 15 to 20 minutes at room temperature, most of the red blood cells had sedimented to the bottom of the tube. The supernatant containing nucleated cells was removed, the cells washed twice in TCM and finally resuspended in TCM to a volume of about 1 ml. Differential count of the washed cells showed a similar distribution of leucocytes to peripheral whole blood.

Staining with monoclonal antibodies. The washed cells were aliquoted into the U-bottomed wells of a Cooke disposable flexi-plate (Dynatech Lab. Ltd, Sussex). The aliquot generally consisted of 1×10^5 nucleated cells but as few as 2×10^4 cells gave satisfactory results on analysis. To each well was added 50 μ l of antisera, and the plate containing the samples was then incubated on ice for 30 minutes. Table 1 gives details of the panel of monoclonal antisera used. The antisera were either hybridoma supernatants or purified immunoglobulin diluted to a concentration so that 50 μ l gave optimal staining of 2×10^5 cells.

After incubation, the plate was centrifuged for 1 minute at 250 g in order to pellet the cells. The supernatant was removed by upturning the micro-titration plate over a sink and gently dispersing the cell pellet by placing the plate on a rotary whirlimixer. To this was added 100 μ l of cold TCM and the washing procedure repeated in this manner 3 times. Then 30 μ l of fluorescein-conjugated sheep anti-mouse immunoglobulin was added to each well as the second layer and the cells were incubated on ice for a further 30 minutes. The cells were then washed 3 times as before and resuspended in 100 μ l cold TCM in LP3 test-tubes. With each test sample, a control well was used with TCM only as the first layer. Previous studies have shown that TCM as the first layer gives no greater background staining than an irrelevant monoclonal antibody.

Fluorescence analysis. A mixture (200 μ l) containing 4 drops of Zapoglobin in 20 μ l Isoton II (Coulter Electronics Ltd) was added to each tube immediately

Table 1 Antisera used

Antisera	Equivalent reagent	Specificity
UCHT1 (T28)	OKT3	Peripheral T cells and some monocytes ¹⁸
UCHT4 (C3)	OKT8/Leu 2A	Suppressor/cytotoxic subset of T cells and some thymocytes (P C L Beverley, 1982, personal communication)
Leu 3A	OKT4	Helper/inducer T cell subset and some thymocytes ¹⁹
2A1	—	Non-polymorphic determinant of HLA A + B ²⁰
Anti-HLe-1	—	All haemopoietic cells with the exception of recognisable erythrocytes and their precursors and platelets ²¹
TG 1	—	Granulocytes and their precursors ²²
UCHM1 (C7)	—	Majority of monocytes (P C L Beverley, 1982, personal communication)
Anti HIg	—	Polyclonal sheep anti-human antiserum which reacts with all immunoglobulin classes (P C L Beverley, 1982, personal communication)

Leu 3A kindly provided by Becton Dickinson, Mountain View, California. Other antisera kindly provided by Dr P C L Beverley, Human Tumour Immunology Group, ICRF, University College Medical School, London.

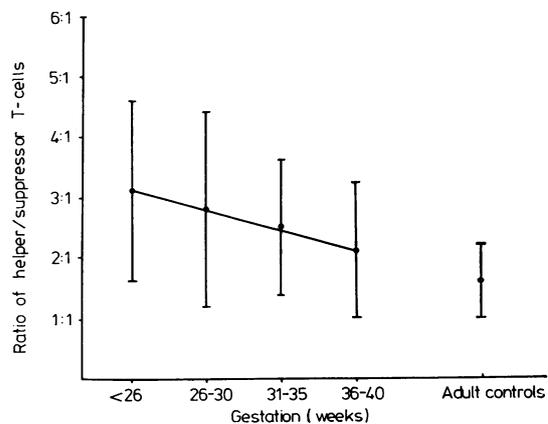


Figure Helper and suppressor T-cell ratios in preterm infants and adult controls. Ratios expressed as mean \pm 1 SD for each gestational group. Regression equation $y = 4.275 - 0.053 \times r = 0.263$ $0.05 > P > 0.02$.

before analysis in order to lyse the red cells. The samples were then analysed on a fluorescent-activated cell sorter (FACS IV, Becton Dickinson) using in general 200 milli Watts laser power, a scatter gain of 8/0.8 with a threshold of 6, a fluorescent gain of 16/1, and a PMT voltage of 700 V. The windows for analysis were set by examination of the fluorescent histograms of UCH T1 and control samples. The percentage of positive cells in the control sample (generally fewer than 6%) was subtracted from the values obtained with all antisera except the combination 2A1 + 2D1 which stains all white cells but not nucleated red cells (generally 95–100%) (Figure). The values obtained were then expressed as a percentage of the total 2A1 + 2D1 positive cells, so giving a differential leucocyte count. The total white cell count of the whole blood was determined on a Coulter S-plus so that absolute numbers of different leucocyte subsets could be estimated.

Double staining. Double staining for suppressor- or cytotoxic-related antigen and helper- or inducer-related antigen was performed with UCHT4 which is an IgG2 antibody and Leu 3A which is an IgG1 antibody. Cells were stained simultaneously with both antisera in the first layer as described above. Immunoglobulin subclass specific, rhodamine-labelled anti IgG, and fluorescein labelled anti IgG (Nordic Immunological Laboratories, Netherlands) were used together in the second layer. Double

staining was determined using a Zeiss fluorescence microscope.

Results

AGA infants. The normal range of total T-lymphocytes, T-cell subsets, and B-lymphocytes has been determined in 54 normal healthy infants (gestation range 23 to 42 weeks), mean birthweight 2073 g (range 610 to 4880) and is shown in Table 2. There was no significant difference between absolute numbers of total T-cells, suppressor or cytotoxic T-cells, helper or inducer T-cells, or B-cells in preterm infants compared with term ones (Table 3). The absolute values obtained in 10 normal healthy adult controls were all significantly lower than those obtained in the preterm and term infants ($0.01 > P > 0.001$). The ratio of helper or inducer to suppressor or cytotoxic T-cells varied greatly in the newborn population, ranging from 15:1 to 0.6:1 with the mean for the whole appropriate for gestation (AGA) newborn population being 2.8:1. In the control adults the same ratio had a range of 2.6:1 to 0.8:1 and a mean ratio of 1.6:1. Applying the same whole blood technique to samples obtained at diagnostic fetoscopy in the 2nd trimester we have previously shown⁶ the ratio to have a mean value of 3.6:1 (gestation 14 to 26 weeks). This fetal helper or suppressor ratio of 3.6:1 is significantly higher than our adult control ratio of 1.6:1 ($P < 0.001$). The Figure illustrates the decreasing ratio with

Table 2 Normal values of lymphocyte subsets for AGA neonates in the first week of life expressed in absolute numbers $\times 10^9/l$. (Mean ± 1 SD shown)

Gestation (weeks)	No	Total T-cell	Suppressor T-cells	Helper T-cells	B cells
<26	3	3.0 (± 1.4)	1.3 (± 0.9)	1.8 (± 0.6)	0.6 (± 0.8)
26–27	8	5.6 (± 3.0)	1.7 (± 1.0)	4.5 (± 2.6)	1.9 (± 2.0)
28–29	2	2.3 (± 1.2)	0.5 (± 0.1)	1.7 (± 0.7)	0.6 (± 0.3)
30–31	4	4.2 (± 1.6)	1.2 (± 0.7)	3.0 (± 1.2)	1.0 (± 1.4)
32–33	10	4.2 (± 1.7)	1.4 (± 0.4)	3.0 (± 0.8)	1.1 (± 0.9)
34–35	7	5.3 (± 2.6)	1.8 (± 0.7)	4.0 (± 2.1)	1.2 (± 0.9)
36–37	8	4.5 (± 0.9)	1.4 (± 0.8)	3.4 (± 0.7)	1.2 (± 0.8)
38–39	2	6.7 (± 2.7)	0.8 (± 0.4)	4.2 (± 2.0)	3.2 (± 2.0)
40	10	5.2 (± 2.7)	2.0 (± 1.3)	3.8 (± 2.4)	0.9 (± 0.9)
All gestations	54	4.7 (± 2.2)*	1.5 (± 0.9 **)	3.5 (± 1.8 **)	1.2 (± 1.0 **)
Adult controls	10	1.6 (± 0.4)	0.7 (± 0.2)	1.1 (± 0.5)	0.3 (± 0.3)

P values obtained by Student's *t* test on means of whole newborn population and adult controls. * $P < 0.001$, ** $P < 0.01$.

Table 3 Lymphocyte subsets of AGA term compared with preterm infants showing percentages of total white cell count and absolute numbers per $10^9/l$. (Mean ± 1 SD shown)

Group		Total T-cells	Suppressor T-cells	Helper T-cells	B-cells
Term (n=10)	Percentage total leucocytes	34 (± 17)	13 (± 7)	24 (± 13)	5 (± 4)
	Absolute number $\times 10^9/l$	5.2 (± 2.7)	2.0 (± 1.3)	3.8 (± 2.4)	0.9 (± 0.9)
Preterm (n=38)	Percentage total leucocytes	34 (± 15)	11 (± 6)	25 (± 13)	8 (± 6)
	Absolute number $\times 10^9/l$	4.5 (± 2.1)	1.5 (± 0.8)	4.7 (± 0.8)	1.3 (± 1.2)

No significant difference found between term and preterm infants.

Table 4 Lymphocyte subsets of SGA compared with AGA infants of all gestations and preterm AGA infants showing percentages of total white cell count and absolute numbers per 10⁹/l. (Mean \pm 1 SD shown)

Group		Total T-cells	Suppressor T-cells	Helper T-cells	B-cells
SGA (n=10)	Percentage total leucocytes	34 (\pm 18)	13 (\pm 8)	20 (\pm 12)	4 (\pm 2)
	Absolute number \times 10 ⁹ /l	3.1 (\pm 1.2)	1.2 (\pm 0.6)	1.9 (\pm 0.9)	0.4 (\pm 0.2)
AGA (n=54)	Percentage total leucocytes	34 (\pm 16)	11 (\pm 6)	25 (\pm 13)	7 (\pm 6)
	Absolute number \times 10 ⁹ /l	4.7 (\pm 2.2)**	1.6 (\pm 0.9)	3.5 (\pm 1.8)*	1.2 (\pm 1.0)*
Preterm AGA (n=38)	Percentage total leucocytes	34 (\pm 15)	11 (\pm 6)	25 (\pm 13)	8 (\pm 6)
	Absolute number \times 10 ⁹ /l	4.5 (\pm 2.1)†	1.5 (\pm 0.8)	4.7 (\pm 0.8)**	1.3 (\pm 1.2)**

P values obtained by Student's test on means. *P<0.01, **P<0.02, †P<0.05.

increasing gestation in samples obtained at fetoscopy in our newborn infants and control adult populations.

In a few infants there was a small overlap in cells staining with 2A and 3A, suggesting the presence of doubly-labelled cells. However, a double staining technique using differences in monoclonal antibody subclasses showed double labelling in fewer than 4% of cells. A cortical thymocyte monoclonal antibody (Na 134) was examined in a few cases and was found to be negative. Our previous study of early fetal blood also showed this to be negative.

Samples obtained from infants within 24 hours of birth showed no difference compared with samples taken on days 3 or 7, and the lymphocyte subset distribution did not vary according to the infant's gender.

Four infants were followed with repeat analyses at weekly intervals ranging from 4 to 9 weeks after birth. In each of the 4 infants followed up, there was no consistent pattern of change with increasing postnatal age. The total T-cell numbers varied (either up or down) but the ratio of helper to suppressor cells remained fairly constant throughout the period of observation.

SGA infants. Ten SGA infants, mean birthweight 1654 g (range 680–2430) were studied. Compared with 54 AGA infants of all gestational ages, there was a significant reduction in absolute numbers of total T-lymphocytes, helper or inducer T-cells, and B-lymphocytes in the SGA infants. This is shown in Table 4. When the SGA infants were compared with the AGA preterm infants, this difference in absolute values of T- and B-cells remained significantly lower in the SGA infants. SGA infants also had higher absolute numbers of all T- and B-cells tested compared with adult values (P<0.05), although as stated previously, the SGA infants also had reduced absolute numbers compared with term infants.

Discussion

This study has shown a number of differences

between lymphocyte subsets of newborn infants and normal adults. All newborn infants studied, including preterm, term, and SGA infants, appear to have a highly significant increase in T- and B-lymphocytes compared with adult values. This finding is consistent with the well-known lymphocytosis of early infancy.¹ The adult control values we obtained compare favourably with those estimated by Brown and Greaves who also stressed the importance of using whole blood preparations in order to calculate absolute numbers.⁷ The increase in absolute numbers of lymphocyte subsets in the newborn may easily be missed using techniques expressing results as a percentage of mononuclear or lymphocyte preparations. Fleisher *et al.*⁸ recommended that both percentage and absolute numbers of cells be reported, and they also found an increase in absolute numbers of T- and B-lymphocytes (using E and EAC binding techniques) in younger children. The newborn infant appears to have adequate numbers of phenotypically mature T- and B-lymphocytes, at least from the 2nd trimester onwards, although functional immaturity has been shown.^{8, 9, 10}

The changing ratio of helper or inducer to suppressor or cytotoxic cells with increasing gestational age suggests that the suppressor or cytotoxic cells are reduced in early gestation, but no significant reduction in absolute number of suppressor or cytotoxic cells was found in our preterm population. Hayward and Kurnick¹¹ demonstrated a lower percentage of cells with suppressor phenotype (OKT8+) in newborn infants compared with adults despite the spontaneous suppressor activity of the former. The mean ratio (n=6 neonates) of helper to suppressor cells in their study was 8:1, compared with our ratio (n=54 neonates) of 2.8:1. The reasons for this difference are not clear but may be partially accounted for by the different cell separation procedures used. *In-vitro* experiments suggest that newborn suppressor cells, although possibly reduced in percentage at birth, are capable of rapid proliferation after pokeweed mitogen or phytohaemagglutinin stimulation.¹² Several workers indicate that the suppressor population can also

expand *in vivo* during the first weeks of life after antigen challenge—such as severe infection or exchange transfusion.^{12 13}

In our study SGA infants have a significantly smaller number of total T-cells, helper or inducer T-cells, and B-cells but not of suppressor or cytotoxic T-cells, than either the AGA group of all gestations or the preterm AGA babies. A reduced number and percentage of T-lymphocytes defined by rosetting with sheep erythrocytes has been previously demonstrated in intrauterine growth retardation by several workers,^{14 15} and impaired cutaneous delayed hypersensitivity and lymphocyte mitogen responses correlate with the reduction in T-lymphocytes in such infants. The percentage of B-lymphocytes has been reported previously to be similar in growth-retarded and age-matched control infants, but the absolute number of B-cells was significantly less in infants with intrauterine growth retardation.¹⁶ The finding of reduced levels of IgG in growth-retarded neonates¹⁷ is consistent with the decreased number of antibody-producing cell precursors and the deficiency of helper or inducer T-cells demonstrated in this study may be significant.

We are grateful for the use of the fluorescent activated cell sorter provided by the Medical Research Council.

R M T was funded by a Research Training Fellowship from Action Research for the Crippled Child. D C L is a Wellcome Research Fellow.

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Received 27 September 1982