IMMUNOPHENOTYPING OF LYMPHOCYTES IN HEALTHY AND IMMUNODEFICIENT CHILDREN

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Immunophenotyping of lymphocytes in healthy and immunodeficient children / Esther de Vries. - Rotterdam : Erasmus Universiteit Rotterdam, Afdeling Immunologie. - ill.
Proefschrift Rotterdam. - Met lit. opg.
ISBN 90-73436-47-8
Trefwoorden: Immunofenotypering, immunodeficiëntie, lymfocyten, kinderen.
Key words: Immunophenotyping, immunodeficiency, lymphocytes, children.

IMMUNOPHENOTYPING OF LYMPHOCYTES IN HEALTHY AND IMMUNODEFICIENT CHILDREN

IMMUNOFENOTYPERING BIJ GEZONDE KINDEREN EN KINDEREN MET EEN AFWEERSTOORNIS

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof. dr. P.W.C. Akkermans M.A.
en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op
woensdag 28 april 1999 om 15.45 uur

door

Esther de Vries

geboren te Amsterdam.

PROMOTIECOMMISSIE:

Promotoren:	Prof. dr. J.J.M. van Dongen Prof. dr. J.M.J.J. Vossen
Overige leden:	Prof. dr. R. de Groot Prof. dr. R. Benner Prof. dr. H.J. Neijens Dr. M.J.D. van Tol

Het uitgeven van dit proefschrift werd mede mogelijk gemaakt door: Abbott B.V., Amgen B.V., Boehringer Ingelheim B.V., Glaxo Wellcome B.V., Merck Sharp & Dohme B.V., Pfizer B.V., Astra Pharmaceutica B.V., Friesland Nutrition en Alk Benelux B.V.

Omslagontwerp: Sascha Jansz, 10 jaar.

Dit proefschrift werd bewerkt binnen de vakgroep Immunologie van de Faculteit der Geneeskunde en Gezondheidswetenschappen, Erasmus Universiteit Rotterdam/Academisch Ziekenhuis Rotterdam, de vakgroep Kindergeneeskunde van de Faculteit der Geneeskunde, Leids Universitair Medisch Centrum, en de vakgroep Kindergeneeskunde van het Sophia Kinderziekenhuis/Academisch Ziekenhuis Rotterdam.

Het proefschrift werd gedrukt door Haveka B.V. te Alblasserdam.

Als schepen elkaar met unverfroren
vaderlandsliefde de grond inboren
van de indonesische archipel

als raketten sneller dan het geluid
afgevuurd alle kanten uit
ons ruimschoots voorzien van kippevel

is er moed voor nodig om ter wereld te komen -
maar zie, de zon schijnt door de bomen
en de meisjes hebben zich mooi gemaakt.

is het geen heerlijke dag vandaag?
de feeën om je wieg zullen goed voor je zorgen
E s t h e r - wees welkom. Goedemorgen!

januari 1962
Wim Wilmink

Aan mijn ouders, Arjan, Sascha en Robin

IMMUNOPHENOTYPING OF LYMPHOCYTES IN HEALTHY AND IMMUNODEFICIENT CHILDREN

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PART I

TECHNICAL ASPECTS OF IMMUNOPHENOTYPING IN CHILDREN

CHAPTER 1

INTRODUCTION

INTRODUCTION

This thesis describes the use of immunophenotyping of lymphocytes in healthy as well as immunodeficient children. Part I describes the applied techniques (Chapters 2 and 3). The experimental work in healthy children is described in Part II (Chapters 4-7) and Part III describes the experimental work in immunodeficient children (Chapters 8-13). A summary and discussion of the work is given in Part IV.

Chapter 2 shortly describes some technical aspects of flow cytometric immunophenotyping of lymphocytes. The advantages of using the lysed whole blood technique with directly conjugated monoclonal antibodies are discussed.

In **Chapter 3** a microassay for immunophenotyping of neonatal and infant lymphocytes is described. This microassay was developed for the study of healthy children during their first year of life, to enable detailed immunophenotyping in a small volume of blood (0.5 to 1.0 ml). In neonatal cord blood samples, contamination of the flow cytometric "lympho-gate" with erythroid cells leads to underestimation of lymphocyte subpopulations. This erythroid contamination can be easily identified with the GpA/CD71/CD45 triple immunostaining, which allows correction for the presence of erythroid cells within the "lympho-gate".

Chapter 4 constitutes the introduction to the immunophenotyping studies in healthy children. Problems and pitfalls encountered in the study of the developing pediatric lymphocyte system are discussed, and literature data on the current knowledge of lymphocyte development in neonates and infants are summarized. The age-related differences in lymphocyte subpopulations and function imply that the available adult reference values cannot be used in children.

In **Chapter 5** the actual sizes (i.e. the absolute counts) of the "naive" and "mature" blood lymphocyte subpopulations are compared between preterm neonates, term neonates, and adults. Unexpectedly, "mature" blood lymphocyte subpopulations appeared to be present in comparable absolute counts in term neonates and adults. However, "naive" lymphocyte subsets occurred in essentially higher absolute counts in term neonates, suggesting that a large pool of "naive" (untriggered) cells is standby in neonates, ready for participation in primary immune responses. This pool of "naive" lymphocytes was not so large in some preterm neonates, which may partly explain their higher susceptibility to infections.

Chapter 6 presents the longitudinal follow-up study of T-lymphocytes, B-lymphocytes, and NK-cells in ten healthy children from birth to one year of age. T- and B-lymphocytes increased at one and six weeks of age, respectively, whereas NK-cells showed a sharp decline directly after birth. These data show that the changes in size of the lymphocyte subpopulations that are found in cross-sectional studies are confirmed in longitudinal studies of individual children.

In **Chapter 7** the longitudinal follow-up of several subpopulations of T-lymphocytes, B-lymphocytes, and NK-cells during the first year of life is shown. Unexpectedly, the CD45RO+ T-lymphocyte count did not change during the first year of life, whereas the CD45RA+ T-lymphocyte count increased markedly, providing a large pool of cells for participation in primary immune responses. CD38 (T- and B-lymphocytes) and CD1c and CD5 (B-lymphocytes) are discussed as possible markers of peripheral immaturity. Finally, NK-cell subpopulations and their possible role before and after birth are discussed.

Chapter 8 gives an introduction to the immunophenotyping studies in immunodeficient children. A stepwise protocol for the diagnosis of immunodeficiency is presented, including an immunophenotyping protocol for clinical use. The role and interpretation of immunophenotyping results in the diagnostic process are discussed.

In **Chapter 9** the use of immunophenotyping in studying the slow reconstitution of lymphocyte subpopulations after pediatric bone marrow transplantation is illustrated. Especially the CD4+ helper T-lymphocytes recovered slowly and reached the p_5 of age-matched reference values at a late stage (> 6 months posttransplant). The unexpected acceleration of the reconstitution of CD4+ helper T-lymphocytes in patients with CMV-reactivation is discussed. The posttransplant development of the absolute counts of CD45RA+, CD45RO+, and CD7- T-lymphocyte counts are discussed in relation to normal ontogeny and the possibility of peripheral expansion of previously primed donor-derived "memory" T-lymphocytes versus newly processed T-lymphocytes via T-lymphopoiesis in the thymus.

In **Chapter 10** the use of immunophenotyping is described for the identification of an unusual $F_c\gamma$Receptor IIIa (CD16) on NK-cells in a patient with recurrent infections. Loss of the epitope for the CD16 (B73.1) monoclonal antibody led to the identification of $F_c\gamma$RIIIA-48H/H homozygosity. The combined use of CD16 and CD56 monoclonal antibodies labeled with the same fluorescent dye, as is often applied in routine immunophenotyping procedures, will leave these homozygotes undiagnosed. This might explain why the $F_c\gamma$RIIIA-48H/H phenotype was not recognized earlier, despite the 8% frequency of the 230A allele in the Caucasian population. The potential causal relation between the unusual $F_c\gamma$Receptor IIIa and the clinical problems in the patient is discussed.

Chapter 11 describes the use of immunophenotyping in the characterization of the T-cell defect found in two siblings with recurrent infections. TCR$\alpha\beta$+ T-lymphocytopenia was found, particularly affecting CD4+/CD45RA+ "naive" helper T-lymphocytes. Potential causes for this T-cell defect are discussed. The importance of comparing immunophenotyping results with age-matched controls is illustrated: V_γ and V_δ usage in the patients were normal in comparison with an age-matched control, but not in comparison with an adult control.

In **Chapter 12** the use of immunophenotyping in the diagnosis of X-linked

hyper IgM syndrome is shown: the activated T-lymphocytes did not show expression of CD40 ligand. Analysis of the underlying mutation in the *CD40 ligand* gene and genetic screening of the family are illustrated. The relationship between genotype and clinical phenotype in patients with X-linked hyper IgM syndrome is discussed.

Chapter 13 describes a girl with agammaglobulinemia and absence of blood B-lymphocytes. Immunophenotyping of the patient's bone marrow cells enables characterization of the blockade in B-lymphocyte development. Potential causes for the severe B-cell defect are discussed.

CHAPTER 2

FLOW CYTOMETRIC IMMUNOPHENOTYPING OF LYMPHOCYTES

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INTRODUCTION

Lymphocytes can easily be recognized morphologically, but immunophenotyping with specific antibodies is needed to identify the major types of lymphocytes and the various lymphocyte subpopulations. Modern flow cytometers in combination with specially designed software programs for analysis of the antibody staining results enable rapid and accurate multiparameter evaluation of large numbers of cells in a short period of time.[reviewed in 1,2]

SAMPLE PROCESSING

In the lysed whole blood technique (LWB), a fixed volume of anticoagulated blood is incubated with antibodies, red blood cells are lysed, and the sample is washed before it is analyzed on the flow cytometer. This LWB technique is currently most commonly used for flow cytometric immunophenotyping, because it requires fewer preparation steps and less sample handling than the previously used methods for staining of mononuclear cells (MNC) obtained after separation over a Ficoll density gradient. Furthermore, the LWB technique in principle does not result in differential loss of specific lymphocyte subpopulations, which has been observed in case of cell separation via density gradients.[3]

Nowadays, virtually all routinely used antibodies are monoclonal antibodies (McAb) directly coupled to fluorochromes, such as fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridin chlorophyll protein (PerCP), or the duochrome PE-cyanin 5 (PE-Cy5). It is important to minimize aspecific binding of McAb through F_c receptors which are expressed on monocytes, granulocytes, NK-cells, and some T- and B-lymphocytes.[4] The LWB technique is less sensitive to aspecific binding, probably because immunoglobulin (Ig) molecules present in the plasma block the F_c receptor binding sites. These Ig molecules are washed away during the density centrifugation procedure for isolation of MNC. Dead cells show increased autofluorescence, potentially leading to incorrect interpretation of staining results. This can be minimized by using fresh samples.

THE FLOW CYTOMETER

Within the flow cytometer (Figure 1) the cells pass one by one through a laser beam. This laser beam excites the fluorochromes, which then emit light of a specific wave length. Photodetectors collect these fluorescent signals via several lens and filter systems. The laser beam is also reflected (scattered) by the cells. The forward scatter (FSC) and side scatter (SSC) are measures of size and irregularity of the cells, respectively, and are also collected by photodetectors. The light signals are then converted into electronic signals, which can be stored and analyzed with a computer using specialized software programs.

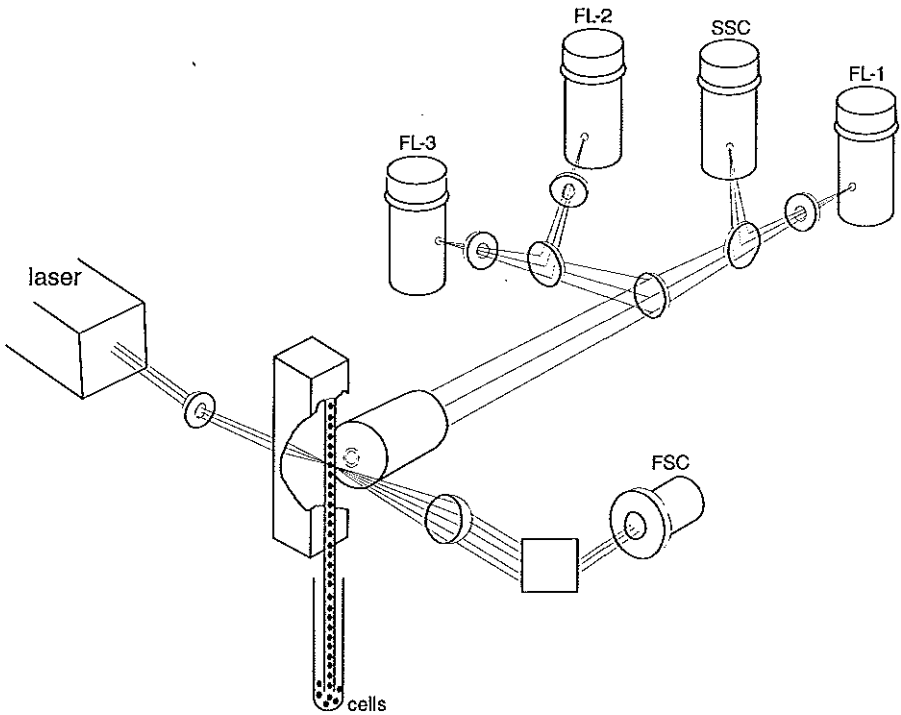

Figure 1. Schematic diagram of a flow cytometer.

DATA ANALYSIS

Data analysis is performed by isolated evaluation of the population of interest, e.g. lymphocytes. This is achieved by electronic gating: the so-called "lympho-gate" is set around the small and regularly shaped lymphocytes in an FSC versus SSC dot plot (Figure 2a). This gating is confirmed with analysis of the CD45 and CD14 staining: lymphocytes are $CD45^{bright}/CD14^{-}$, whereas monocytes are $CD45^{bright}/CD14^{bright}$, and granulocytes are $CD45^{intermediate}/CD14^{intermediate}$. Negative controls are performed with irrelevant McAb of the appropriate class (e.g. IgG1). The staining pattern of the McAb of interest is then analyzed within this "lympho-gate", resulting in data on the intensity of staining as well as the percentages of lymphocyte subpopulations.

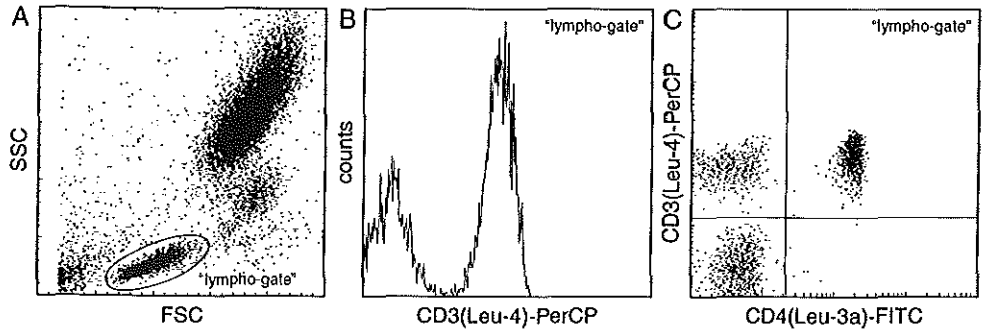

Figure 2. Immunophenotyping of blood T-lymphocytes *A*, FSC versus SSC dot plot with "lympho-gate". *B*, CD3 expression presented in a histogram. *C*, CD3 versus CD4 fluorescence intensity presented in a dot plot.

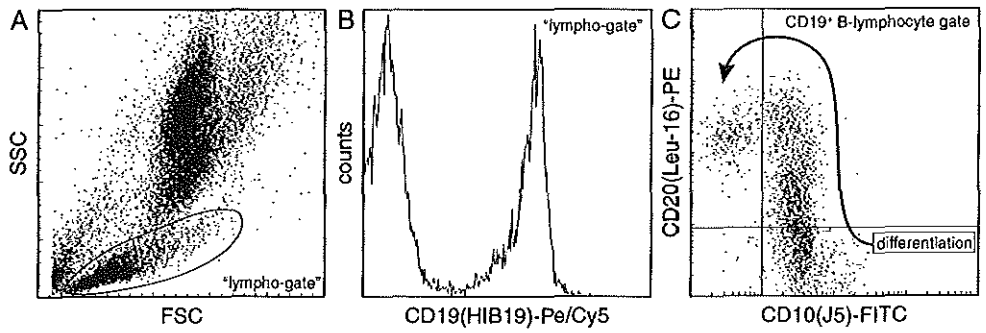

Figure 3. Immunophenotyping of precursor-B-cells in normal bone marrow. *A*, FSC versus SSC dot lot with "lympho-gate". *B*, Histogram of CD19 expression within the "lympho-gate". *C*, CD10 versus CD20 expression within the CD19+ "lympho-gate". The arrow indicates the B-cell differentiation pathway from immature CD19+/CD10++/CD20- precursor-B-cells, to mature CD19+/CD10-/CD20+ B-lymphocytes.

DATA PRESENTATION

A single parameter can be presented with a histogram displaying the fluorescence intensity on the X-axis, and the number of cells on the Y-axis (Figure 2b). Two parameters can be presented simultaneously in a dot plot with the fluorescence intensity of each parameter depicted on the X- and Y-axes; each cell is represented by a single dot (Figure 2c). The positive and negative populations are then discriminated electronically by the operator of the system. This might be difficult when the positivity and negativity for particular markers form a continuous

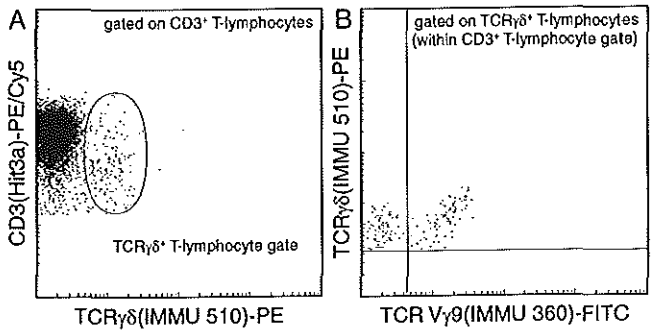

Figure 4. Analysis of TCRγδ+ T-lymphocytes. *A*, After gating on CD3+ T-lymphocytes a second gate is placed on the TCRγδ+ T-lymphocytes. *B*, Detection of Vγ9 expression within the TCRγδ+ T-lymphocyte gate.

spectrum, such as CD10 and CD20 expression during B-cell differentiation in the bone marrow (Figure 3). When more then two fluorochromes are used, it is possible to perform an extra gating step on a subpopulation. This enables analysis of markers within small subpopulations, e.g. V_γ and V_δ gene usage can be analyzed within the TCRγδ+ subpopulation of T-lymphocytes after setting a second gate around the TCRγδ+ cells within the "lympho-gate" (Figure 4). Using the total lymphocyte count obtained with a separate leukocyte count and differentiation, absolute counts of lymphocyte subpopulations can then be calculated.[5]

CHOICE OF REAGENTS FOR IMMUNOPHENOTYPING STUDIES

Flow cytometric immunophenotyping enables quantification of lymphocyte subpopulations, e.g. for diagnosis of CD3+ T-lymphocytopenia in "severe combined immunodeficiency" (SCID), or for follow-up of CD3+/CD4+ helper T-lymphocyte counts in HIV-infected individuals. Alternatively, the presence or absence of a specific antigen can be tested with or without prior activation of the cells, e.g. the absence of CD18 in "leukocyte adhesion deficiency" (LAD), or the absence of CD40 ligand (CD40L) on activated T-lymphocytes in X-linked hyper IgM syndrome (XHIM). Maturation of lymphocytes can be studied with reagents specific for precursor markers such as in studies of precursor-B-cell differentiation in normal bone marrow (Figure 3), which are important for understanding the B-cell differentiation blockade in patients with e.g. X-linked agammaglobulinemia (XLA). Furthermore, "naive" and "memory" markers can identify the maturational status of blood lymphocytes in comparative studies between infants and adults. Flow cytometric immunophenotyping is also used in the diagnosis and classification of leukemias and malignant lymphomas, the differential diagnosis of inflammatory conditions, the isolation of CD34+ cells for stem cell transplantation, and in certain routine measurements like HLA-B27 detection and red blood cell antigen determinations. This thesis focusses on the application of immunophenotyping of lymphocytes in healthy and immunodeficient children.

TABLE 1. Lymphocyte surface antigens studied in this thesis.

CD no.	Antigen	Expression pattern[1]
CD1	common thymocyte antigen	cortical thymocytes (CD1a), subpopulation of B-lymphocytes (CD1c)
CD2	E-rosette receptor	expressed on all T-lymphocytes and most NK-cells
CD3	TCR associated antigen	mature TCR+ T-lymphocytes
CD4	MHC class-II receptor	helper T-lymphocytes; subpopulation of thymocytes; subpopulation of monocytes
CD5	T1 antigen	thymocytes; mature T-lymphocytes; subpopulation of B-lymphocytes
CD7	Tp41 pan-T-cell antigen	almost all T-lymphocytes and NK-cells
CD8	MHC class-I receptor/T8 antigen	cytotoxic/suppressor T-lymphocytes; subpopulation of thymocytes and NK-cells
CD10	common ALL antigen	subpopulation of precursor B-cells
CD14	monocytic antigen	monocytes
CD15	Lewis-X	granlocytes; weak expression by monocytes
CD15s	sialyl-Lewis-X	granulocytes, monocytes, and other myeloid cells; absent in LAD2 patients
CD16	$F_c\gamma$ receptor IIIa	granulocytes; subpopulation of NK-cells
CD18	integrin β_2 chain	most lymphoid and myeloid cells; absent or defect in LAD1 patients
CD19	pan B-cell antigen	precursor-B-cells and mature B-lymphocytes
CD20	mature B-cell antigen	B-lymphocytes; subpopulation of precursor-B-cells
CD21	B-cell antigen/EBV receptor	subpopulation of B-lymphocytes
CD25	α chain of the IL2 receptor	activated T- and B-lymphocytes and NK-cells
CD27	T-cell antigen	mature and activated T-lymphocytes
CD28	CD80(B7/BB1)/CD86(B7-2) receptor	subpopulation of T-lymphocytes
CD29	integrin β_1 chain	mature CD45RO+ T-lymphocytes
CD34	precursor antigen	early precursor cells (lymphoid and myeloid)
CD37	mature B-cell antigen	B-lymphocytes
CD38	T10 antigen	thymocytes; activated lymphocytes; subpopulations of T- and B-lymphocytes
CD40	B-cell antigen	mature B-lymphocytes
CD45	leukocyte common antigen (LCA)	all leukocytes
CD45RA	restricted LCA, isoform of CD45	"naive" T-lymphocytes; B-lymphocytes; NK-cells
CD45RO	restricted LCA, isoform of CD45	"memory" T-lymphocytes; subpopulation of NK-cells
CD56	neural cell adhesion molecule	subpopulations of T-lymphocytes and NK-cells
CD57	human NK-cell antigen	subpopulations of T-lymphocytes and NK-cells
CD69	activation inducer molecule	early activated T- and B-lymphocytes
CD71	transferrin receptor	thymocytes; activated lymphocytes; erythroid precursors
CD79a	mb-1; Igα; Ig associated molecule	precursor-B-cells (Cy expression); B-lymphocytes (Sm expression)
CD79b	B29; Igβ; Ig associated molecule	precursor-B-cells (Cy expression); B-lymphocytes (Sm expression)
CD154	CD40 ligand; gp39	activated T-lymphocytes; absent or defect in XHIM patients
-	β_2 microglobulin (light chain of HLA class-I molecule	all nucleated cells
-	GpA	all erythroid cells
-	HLA-DR; MHC class-II molecule	hematopoietic precursors; B-lymphocytes; activated T-lymphocytes
-	pseudo light chain (VpreB/λ14.1)	precursor B-cells; expressed with CD79a/b + μ heavy chain as preBCR
-	SmIg	B-lymphocytes
-	TCR$\alpha\beta$ ("classical" T-cell receptor)	almost all blood T-lymphocytes
-	TCR$\gamma\delta$ ("alternative" T-cell receptor)	small subpopulation of blood T-lymphocytes

Abbreviations used: ALL = acute lymphatic leukemia, (pre)BCR = (pre) B-cell receptor, Cy = cytoplasmic, EBV = Epstein-Barr virus, GpA = glycophorin A, HLA = human leukocyte antigen, IL-2 = interleukin-2, LAD = leukocyte adhesion deficiency, LCA = leukocyte common antigen, MHC = major histocompatibility complex, SmIg = surface membrane immunoglobulin, TCR = T-cell receptor, XHIM = X-linked hyper IgM syndrome.

[1] As relevant to the discussion in this thesis.

TABLE 2. Intracellular lymphocyte antigens studied in this thesis.

CD no.	Antigen	Expression pattern[1]
CD3	CyCD3	precursor T-cells (cortical thymocytes)
CD79a	CDCD79a(Cymb-1; Cy Igα)	precursor B-cells
CD79b	CyCD79b (Cy B-29; Cy Igβ)	precursor B-cells
-	Cy Igμ (heavy chain)	precursor B-cells of pre-B-cell type
-	Ki 67, early intracellular proliferation marker	proliferating cells during late G1, S, G2, and M phases of the cell cycle
-	pseudo light chain (VpreB/λ14.1), part of preBCR	precursor B-cells
-	TdT (inserts nucleotides at junction sites in Ig and TCR rearrangement)	precursor B-cells and thymocytes

Abbreviations used: (pre)BCR = (pre) B-cell receptor, Cy = cytoplasmic expression, Ig = immunoglobulin, TCR = T-cell receptor, TdT = terminal deoxynucleotidyl transferase.
[1] As relevant to the discussion in this thesis.

It is obvious that the choice of reagents is dependent on the specific diagnostic or research question(s) being asked. Combined staining with CD45 and CD14 McAb and the appropriate control reagents is necessary to enable accurate setting of the "lympho-gate". Enumeration of total T-lymphocytes, B-lymphocytes, and NK-cells provides an important internal control, because taken together this "lympho-sum" should equal the total lymphocyte count. The selection of other markers is directed by the questions studied.

The markers studied in this thesis, the antibodies used for detection, the CD-codes, and the reactivity patterns of the antibodies are summarized in Tables 1 and 2. During six "Leukocyte Typing" conferences, the unique CD ("cluster of differentiation") codes have been assigned to well-characterized McAb to enable comparison of data between laboratories from all over the world. In this thesis the McAb are indicated with their CD-code, supplemented with the hybridoma clone code, and, if applicable, the fluorochrome they are conjugated to. The McAb used in this thesis are listed in the respective chapters.

REFERENCES

1 Fleisher TA. Immunophenotyping of lymphocytes by flow cytometry. *Immunol Allergy Clin* 1994;14:225-240.
2 Van Dongen JJM, Groeneveld K, Adriaansen HJ, Hooijkaas H (eds). Immunofenotypering in de diagnostiek: indicatiestellingen, uitvoering en interpretatie. Dept. of Immunology, Erasmus University Rotterdam, Rotterdam, 1994.
3 Kutvirt SG, Lewis SL, Simon TL. Lymphocyte phenotypes in infants are altered by separation of blood on density gradients. *Brit J Biomed Science* 1993;50:321-328.
4 Van de Winkel JG. Human IgG Fc receptors. *Int Rev Immunol* 1997;16:29-55.
5 Comans-Bitter WM, De Groot R, Van den Beemd MWM, Neijens HJ, Hop WCJ, Groeneveld K, Hooijkaas H, Van Dongen JJM. Immunophenotyping of blood lymphocytes in childhood: Reference values for lymphocyte subpopulations. *J Pediatr* 1997;130:388-393.

CHAPTER 3

CORRECTION FOR ERYTHROID CELL CONTAMINATION IN MICROASSAY FOR IMMUNOPHENOTYPING OF NEONATAL LYMPHOCYTES

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Arch Dis Child, 1999, in press

ABSTRACT

Immunophenotyping of blood lymphocyte subpopulations in neonates and young infants is hampered by the limited amount of blood that can be collected. Contamination of the flow cytometric "lympho-gate" by normoblasts and unlysed erythrocytes and therefore the underestimation of the relative frequencies of lymphocyte subpopulations interferes with the precise calculation of absolute counts.

A microassay was developed by adapting the lysed whole blood technique. Triple immunostaining in a single antibody staining step was used to reduce washing steps and cell loss. Introduction of a triple staining for CD71 (expressed by erythroid precursors), glycophorin A (GpA, expressed by all erythroid cells), and CD45 (expressed by all leukocytes) permitted the relative frequencies of normoblasts (CD71+/GpA+/CD45- population) and unlysed erythrocytes (CD71-/GpA+/CD45- population) to be identified and measured. Particularly high frequencies of erythroid cells were found (median: 31%) in cord blood samples from preterm neonates. These erythroid cells disappear rapidly by 1 week of age, also in preterm neonates. The relative frequencies of erythroid cells can be used to calculate correct lymphocyte subpopulation values. Using only 0.5-0.8ml of blood, this microassay would also be suitable for rapid prenatal immunodiagnosis of congenital immunodeficiencies.

INTRODUCTION

Immunophenotyping of blood lymphocyte subpopulations is an important tool in the diagnosis and follow-up of children with congenital immunodeficiencies, HIV-infection or other immune disorders. Detailed analysis and quantitation of lymphocyte subpopulations is also needed for investigation of age-related maturational processes within the immune system in childhood. Immunophenotyping of blood lymphocytes used to be performed after density gradient separation, but the lysed whole blood technique is preferred now because it prevents selective cell loss, requires smaller amounts of blood, and results in more accurate determination of absolute lymphocyte counts.[1-3]

In neonates and young infants, several problems are encountered in flow cytometric immunophenotyping of blood lymphocytes. The limited blood volume in these children and technical difficulties in venepuncture restrict the amount of blood that can be collected. In neonates the flow cytometric "lympho-gate" can be contaminated with normoblasts and unlysed erythrocytes (Figure 1).[4] Normoblasts are comparable in size and surface membrane to lymphocytes and thus have similar flow cytometric forward scatter (FSC) and side scatter (SSC) characteristics. Neonatal erythrocytes are relatively resistant to osmotic lysis and some of them are sufficiently large to localize in the flow cytometric "lympho-gate".[4] The erythroid cell contamination hampers the determination of the relative frequencies of lymphocyte subpopulations and thus calculation of absolute counts

from the total white cell count, which is generally determined as the total nucleated cell count.[3] In neonates, the presence of normoblasts can also interfere with the determination of the white cell count, because normoblasts are nucleated, and are therefore included in the total count.

To overcome these difficulties, we developed a lysed whole blood microassay with triple immunostaining for the identification of erythroid cells and correction for erythroid cell contamination within the "lympho-gate".

MATERIALS AND METHODS

Twenty-one neonatal cord blood samples were collected by venepuncture immediately after clamping of the cord. The blood was kept at room temperature until labeling with monoclonal antibodies was performed within 12 hours of sampling. Peripheral blood was drawn by venepuncture from nine healthy adult volunteers between 20 and 40 years of age, and at 1 week of age from 14 neonates whose cord blood had been studied previously. Ethylene-diamino-tetra-acetate (EDTA) was used as an anti-coagulant.

Informed consent was obtained according to the guidelines of the Medical Ethics Committee of the Erasmus University Rotterdam / University Hospital Rotterdam in all cases.

The following monoclonal antibodies were used: CD3 (Leu-4a), CD4 (Leu-3a), CD8 (Leu-2a), CD16 (Leu-11c), CD19 (Leu-12), CD56 (Leu-19), CD71 (anti-transferrin receptor), CD45 (HLE1), IgG1 and IgG2 (isotype controls) (Becton Dickinson, San Jose, CA), CD14 (My4), CD19 (B4) (Coulter, Hialeah, FL), CD3 (Hit3a), glycophorinA (GpA) (GA-R2), (Pharmingen, San Diego, CA), CD15 (CLBgran2) (Central Laboratory of the Blood Transfusion Service, Amsterdam, Holland). All monoclonal antibodies were conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), or the duochrome PE-Cyanin5 (PE-Cy5).

Blood samples (100, 40, 20, or 10 µl) were incubated with titrated monoclonal antibodies for 10 minutes at room temperature. After two washes with phosphate buffered saline containing 0.1% NaN_3 and 0.5% bovine serum albumin, erythrocytes were lysed with Lysing Solution (Becton Dickinson) according to the manufacturer's instructions. Before lysis the cell pellet was loosened to prevent incomplete lysis of erythrocytes.

The samples were analyzed using a FACScan flow cytometer (Becton Dickinson). Instrument settings were determined with cells labelled with FITC, PE, and PE-Cy5 conjugated monoclonal antibodies and unstained cells. After gating of lymphocytes on the basis of FSC and SSC (checked by CD14, CD15, and CD45 monoclonal antibody staining), 8000 events were acquired. The data were analyzed using FACScan software (Becton Dickinson) in dot plots, with optimal quadrant setting checked by histogram analysis, if necessary.

Leukocyte count and differentiation

The total nucleated cell count was determined on an H1 Technicon hemocytometer (Bayer, Tarrytown, NY). The relative frequency of normoblasts was carefully determined by manual differentiation of at least 400 nucleated cells. The total leukocyte count was determined according to the following formula:

$$\text{total nucleated cell count} \times (100 - \text{percentage of normoblasts}) \times 10^{-2}$$

The relative frequencies of lymphocyte subpopulations were calculated according to the following formula:

$$\frac{\text{percentage of population within the "lympho-gate"} \times 10^{2}}{100 - (\text{percentage of normoblasts plus percentage of unlysed erythrocytes within the "lympho-gate"})}$$

The Wilcoxon rank sum test or Friedman's test were used to compare two or more than two different techniques used in one blood sample, respectively (α = 0.05).

RESULTS

Development of microassay

We incubated 100, 40, 20, and 10 µl of blood with monoclonal antibodies. When using 20 µl of blood, at least 100,000 nucleated cells remained available after completion of the staining procedure in all samples tested, as determined by use of a cell counter (Coulter Counter; Hialeah, FL). However, when using 10 µl of blood, major and variable cell loss was observed. Relative frequencies of lymphocyte subpopulations determined in parallel with either 100 µl or 20 µl of blood per test tube gave fully comparable results in five separate neonatal blood samples. Therefore, 20 µl of blood incubated with 20 µl of each monoclonal antibody per test tube was used in all further experiments.

The determination of the relative frequencies of lymphocyte subpopulations by either double or triple immunostaining was compared in one adult and six cord blood samples - for example, triple CD4/CD8/CD3 *vs* double CD4/CD8, CD4/CD3 and CD8/CD3 immunostaining. When using triple immunostaining, monoclonal antibodies were incubated consecutively, with each incubation followed by two washes, or all together. No significant differences were found between the relative frequencies of lymphocyte subpopulations, as determined by the different staining methods. Therefore, in all further experiments only triple immunostaining was used with incubation of all three fluorochrome conjugated monoclonal antibodies simultaneously to speed up the microassay and minimize cell loss due to multiple washes.

Table 1. Composition of "lympho-gate" in neonatal and adult blood samples.

"Lympho-gate" populations	Preterm neonates (n=6)			Term neonates (n=15)			The same neonates at one week of age (one preterm and 13 term neonates)			Adults (n=9)		
	Mean	Median	Range	Mean	Median	Range	Mean	Median	Range	Mean	Median	Range
"Lympho-sum" [b]	57[a]	61	24-78	85	86	67-95	95	96	92-98	98	98	97-100
CD14+ monocytes	2	2	1-2	1	1	0-3	1	1	0-2	1	1	0-2
CD15+ granulocytes	1	1	0-1	1	1	0-1	1	1	0-1	0	0	0-1
CD71+/GpA+/CD45- normoblasts	31	29	8-57	10	7	3-28	0	0	0-1	0	0	0-0
CD71-/GpA+/CD45- unlysed erythrocytes	4	3	2-5	2	1	0-11	2	1	0-8	1	1	0-2

[a] All numbers represent percentages
[b] "Lympho-sum = CD19+ + CD3+ + CD3-/CD16/56+ (B + T + NK) without correction for the erythroid cell contamination of the "lympho-gate".

TABLE 2. Illustration of erythroid cell contamination bias in two cord blood samples.

Gestational age	Erythroid cells within the "lympho-gate"		CD3+ T-lymphocytes		CD19+ B-lymphocytes		CD16/56+CD3- NK-cells		"Lympho-sum"	
	Normoblasts	Unlysed erythrocytes	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected
29 weeks	44[a]	5	28	54	18	36	10	20	56	110
35 weeks	40	3	24	43	10	17	17	30	51	89

[a] All numbers represent percentages
[b] Without correction for the erythroid contamination of the "lympho-gate".
[c] With correction for the erythroid contamination of the "lympho-gate".

Detection of erythroid cell contamination

When a scatter gate is set around the lymphocyte population, it is generally not possible to exclude fully other contaminating cells without excluding lymphocytes. However, as long as the sum of the relative frequencies of B-lymphocytes, T-lymphocytes, and NK-cells (the so-called "lympho-sum") is at least 95% (or 0.95), the "lympho-gate" is often considered to be placed correctly. The remaining events within the "lympho-gate" are monocytes, granulocytes, and unidentified events, which generally consist of dead cells and debris. In our neonatal cord blood samples the "lympho-sum" rarely reached 95% of the "lympho-gate" (Table 1). In preterm neonates, in particular, the "lympho-sum" seemed to be far too low. This was caused by a relatively high frequency of unidentified events (Figure 1).

Normoblasts express CD71 and GpA, whereas erythrocytes express only GpA.

In contrast, leukocytes express CD45, unlike normoblasts and erythrocytes. Therefore, CD71+/GpA+/CD45-cells within the "lympho-gate" can be regarded as normoblasts, whereas unlysed erythrocytes have the CD71-/GpA+/CD45- immunophenotype.[5] By triple immunostaining with CD71-FITC, GpA-PE, and CD45-PE-Cy5, these unidentified events could be identified as contamination by normoblasts and in some samples by unlysed erythrocytes as well (Figure 2; Table 1). The FSC and SSC of these normoblasts and unlysed erythrocytes was checked ("back-gating"), and was found to be relatively low, as expected for these cells (Figures 2c and 2d).

A small percentage of events remained unidentified - that is, they were neither CD45+ leukocytes, nor CD71+/GpA+/CD45- normoblasts, nor CD71-/GpA+/CD45- unlysed erythrocytes (left lower quadrant of dot plot in Figure 2b). These events may represent dead cells and debris.

Erythroid cell contamination in neonatal blood

CD71/GpA/CD45 staining was used to determine the relative counts of normoblasts and unlysed erythrocytes and to calculate the relative counts of lymphocyte subpopulations. The differences in relative frequencies of lymphocyte subpopulations in neonatal cord blood with and without a correction for normoblasts and unlysed erythrocytes are shown in Table 2 for two samples. Normoblasts were not found by manual differentiation or flow cytometric immunophenotyping in blood samples from the same neonates at 1 week of age nor in blood samples from adults, but unlysed erythrocytes were still present in some samples at 1 week of age (Table 1).

DISCUSSION

We have developed a microassay for flow cytometric determination of blood lymphocyte subpopulations in neonates and young infants by adapting the lysed whole blood technique using one step triple immunostaining. When only double immunostaining is available, more blood is needed and less detailed results are obtained - for example, double-positive CD45RA+/CD45RO+ CD3+ T-lymphocytes cannot be identified. This microassay needs only about 0.4ml of blood to determine T-lymphocyte subpopulations for monitoring HIV-infected infants, and 0.5 to 0.8ml of blood is sufficient for detailed evaluation of infants with suspected congenital immunodeficiencies. Extensive protocols for research purposes can also be performed, because 1.0ml of blood is sufficient for more than 40 triple immunostainings. These are acceptable volumes in infants and even in preterm babies.

Normoblasts and unlysed erythrocytes can be present in considerable numbers in neonatal samples.[4] The presence of normoblasts cannot be prevented by technical measures, but strict adherence to a rigorous lysing protocol can minimize the number of unlysed erythrocytes in a sample. Therefore,

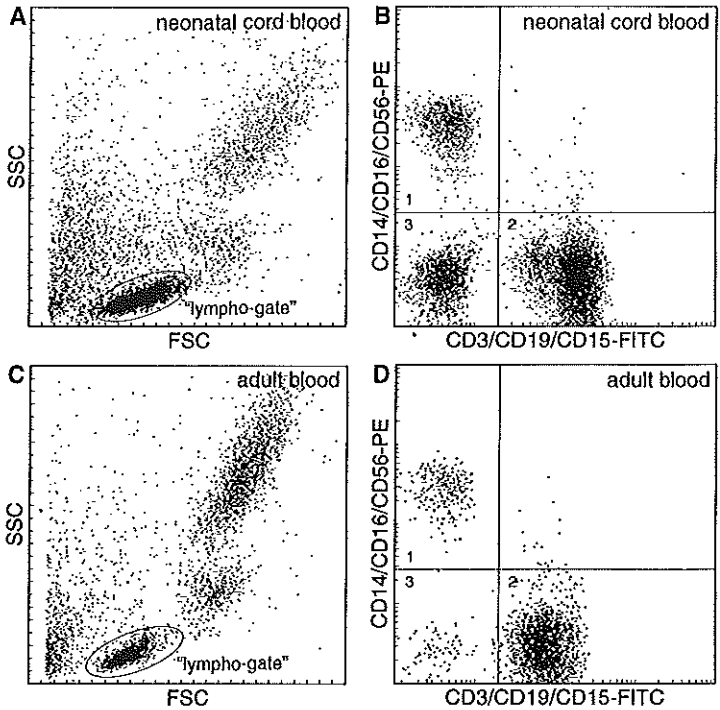

Figure 1. Flow cytometric immunophenotyping of neonatal cord blood lymphocytes reveals a high frequency of unidentified events within the "lympho-gate". *A*, "lympho-gate" setting in neonatal cord blood, based upon FSC/SSC characteristics. *B*, Composition of the "lympho-gate" in neonatal cord blood: many unidentified events and relatively high numbers of NK-cells were observed. *C*, "Lympho-gate" setting in adult blood. *D*, Composition of the "lympho-gate" in adult blood: virtually no unidentified events and relatively low numbers of NK-cells were observed. Quadrant 1: CD14+ contaminating monocytes, and CD3-/CD16+/CD56+ NK-cells. Quadrant 2: CD3+ T-lymphocytes, CD19+ B-lymphocytes, and CD15+ contaminating granulocytes. Quadrant 3: Unidentified events.

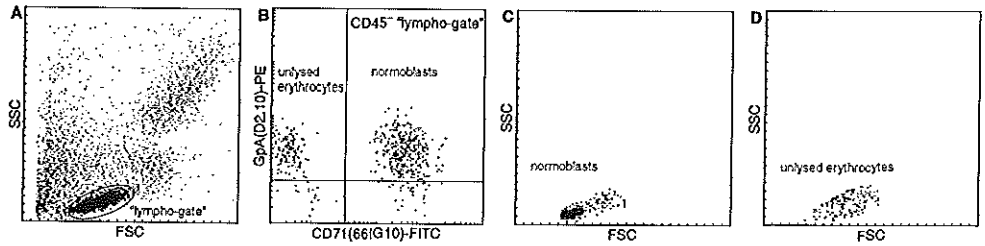

Figure 2. Triple staining for CD71, GpA, and CD45 identifies normoblasts and unlysed erythrocytes within the "lympho-gate". *A*, "Lympho-gate" setting. *B*, CD71+/GpA+/CD45- population of normoblasts and the CD71-/GpA+/CD45- population of unlysed erythrocytes within the CD45- "lympho-gate". *C*, "Back-gating" of CD71+/GpA+/CD45- normoblasts into the "lympho-gate". *D*, "Back-gating" of CD71-/GpA+/CD45- unlysed erythrocytes into the "lympho-gate".

identification of both cell populations is of interest. Using triple immunostaining for CD71, GpA, and CD45, we were able to identify both normoblasts

(CD71+/GpA+/CD45- cells) and unlysed erythrocytes (CD71-/ GpA+/CD45- cells) within the "lympho-gate", and to measure their relative frequencies. When only double immunostaining is available, the GpA+/CD45- cells can be used to measure the relative frequency of erythroid cells, but normoblasts and unlysed erythrocytes cannot be identified separately. The relative frequencies of erythroid cells can be used to calculate the correct relative frequencies of blood lymphocyte subpopulations. This is especially important in preterm neonatal and prenatal blood samples, where the relative frequencies of normoblasts and unlysed erythrocytes can be high (Tables 1 and 2),[6] and the frequencies of lymphocyte subpopulations can be underestimated. This can be confusing if a congenital immunodeficiency is suspected. The erythroid cell contamination of the "lympho-gate" rapidly disappears after birth and is virtually absent by 1 week of age (Table 1).

In conclusion, we have developed a fast and easy to use lysed whole blood microassay for immunophenotyping neonatal and infant blood lymphocyte subpopulations. It requires only a small volume of blood (20 µl per test tube), and offers a method to correct for the presence of normoblasts and unlysed erythrocytes in neonatal samples. It would also be suitable for rapid prenatal immunodiagnosis of congenital immunodeficiencies when genetic markers are not available.[7]

REFERENCES

1 Remy N, Oberreit M, Thoenes G, Wahn U. Lymphocyte subsets in whole blood and isolated mononuclear leukocytes of healthy infants and children. *Eur J Pediatr* 1991;150:230-233.

2 Kutvirt SG, Lewis SL, Simon TL. Lymphocyte phenotypes in infants are altered by separation of blood on density gradients. *Br J Biomed Sci* 1993;50:321-328.

3 Comans-Bitter WM, De Groot R, Van den Beemd MWM, Neijens HJ, Hop WCJ, Groeneveld K, Hooijkaas H, Van Dongen JJM. Immunophenotyping of blood lymphocytes in childhood: Reference values for lymphocyte subpopulations. *J Pediatr* 1997;130:388-393.

4 Oski FA. The erythrocyte and its disorders. In: Nathan DG, Oski FA, eds. *Hematology of infancy and childhood.* Philadelphia: Saunders, 1993:18-43.

5 Loken MR, Shah VO, Dattilio KL, Civin CI. Flow cytometric analysis of human bone marrow: I. Normal erythroid development. *Blood* 1987;69:255-263.

6 Letsky EA. Appendix I: Haematological values in the newborn. In: Roberton NRC, ed. *Textbook of neonatology.* Edinburgh: Churchill Livingstone, 1992:1231.

7 WHO Scientific Group. Primary immunodeficiency diseases. *Clin Exp Immunol* 1995;99(S1.2):1-24.

PART II

LYMPHOCYTE SUBPOPULATIONS IN HEALTHY CHILDREN

CHAPTER 4

ANALYZING THE DEVELOPING LYMPHOCYTE SYSTEM OF NEONATES AND INFANTS

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Eur J Pediatr, 1999, in press

ABSTRACT

Technical developments in immunophenotyping and function testing have greatly facilitated studies on the developing lymphocyte system in the past decade and contributed to a better interpretation of the data obtained in these studies. This is important for the correct interpretation of data obtained in pediatric patients with possible immunological diseases. The age-related differences in lymphocyte subpopulations and function imply that the available adult reference values can not be used in children.

In this review we give an outline of the technical developments, their influence upon the interpretation of data, and the available literature about age-related changes in the developing lymphocyte system.

INTRODUCTION

Immunophenotyping of blood lymphocyte subpopulations and assays of lymphocyte function are important tools in the diagnosis and follow-up of children with immunodeficiencies and other immune disorders. Correct interpretation of the results obtained in patients requires knowledge of the normal development of the immune system during the first years of life. In this review we give an outline of this rapidly evolving field.

Several differences between lymphocyte subpopulations obtained by immunophenotyping in healthy neonates, infants and adults have been reported.[8,14] In addition, in neonates and infants, lower levels of lymphocyte function as compared to adults have been described.[29] This implies that the available adult reference values cannot be used in children.

The decreased levels of lymphocyte function in neonates and infants should not be regarded as a deficiency. Healthy neonates and infants are immunocompetent, but, because of the virgin status of their immune system, they do react differently to antigens and are less efficient in coping with some pathogens.[29] Their lymphocytes undergo maturational changes that are essential for normal development: the virgin immune system is being educated.

In the past decades, many reports have been published on the development of the lymphocyte system during the first years of life. Over time, it has become clear that several problems and pitfalls are encountered in the analysis of lymphocytes in neonates and infants, which have led to erroneous conclusions. Therefore, interpretation of the obtained results has been adapted gradually.

PROBLEMS ENCOUNTERED IN PEDIATRIC STUDIES

Most studies on the human immune system have been performed in adults,

and many questions concerning lymphocyte development in children are not yet (fully) answered. This is mainly due to the fact that it is more difficult to obtain blood from healthy children than from healthy adults. Also, the volume of blood that is needed for these studies can pose a practical problem. Therefore, most pediatric studies have been performed on neonatal cord blood, which can easily be obtained in larger quantities. However, results from cord blood are of limited value for understanding lymphocyte development at later time points in infancy and childhood. Another problem when using cord blood for flow cytometric immunophenotyping is the presence of normoblasts in the circulation before and shortly after birth (see below).[35]

TECHNICAL DEVELOPMENTS

Initially, immunophenotyping of blood lymphocyte subpopulations was only performed on mononuclear cell (MNC) fractions obtained after separation of the blood samples over a Ficoll density gradient; such MNC fractions mainly contain monocytes and lymphocytes. Currently, absolute counts of lymphocyte subpopulations can be determined more accurately by means of the flow cytometric lysed whole blood technique, which allows analysis of unseparated blood samples after osmotic lysis of the erythrocytes. This technique also prevents selective cell loss in some subpopulations, as seen in Ficoll density gradients.[26] Furthermore, virtually all polyclonal antisera have been replaced by well-defined monoclonal antibodies (McAb), many of which can be obtained as fluorochrome conjugates. This minimizes aspecific binding and allows easy immunostaining for detailed analysis of lymphocyte subpopulations by multiparameter flow cytometry. Generally, in flow cytometry the staining pattern of combinations of two to three fluorochrome-conjugated antibodies are analyzed within the "lympho-gate", set according to the lymphocytes' forward scatter (FSC) and side scatter (SSC) characteristics, which are regarded as measures for size and irregularity of the cells, respectively (Figure 1).[30] Recently, reference values for relative frequencies and absolute counts of the main lymphocyte subpopulations were obtained with this technique for all pediatric age groups.[8]

The determination of lymphocyte activation and proliferation after mitogen stimulation has developed from overall measurement of radioactively labeled thymidine uptake in MNC fractions (a measure of DNA-synthesis) to non-radioactive flow cytometric methods, such as calcium influx measurement,[17] detection of activation-induced molecules like cluster of differentiation (CD) 69,[5] and measurement of expression of intracellular proliferation antigens like Ki67 at the single cell level (Figure 2).[20,22] Furthermore, tests using interleukins (IL) (e.g. IL2), specific antigens (e.g. tetanus toxoid), and antibodies which recognize specific co-stimulatory molecules on T- and B-cells (e.g. CD3, CD2, CD28, and CD40 proteins), have been developed for lymphocyte stimulation, enabling a more detailed analysis of defects in lymphocyte function.[17] These tests all show great variability and reference values for children have not yet been established. Therefore, cautious interpretation of results is warranted, preferably with age-

matched controls run in parallel.

The determination of soluble cytokines has improved as well, because most cytokines can nowadays be measured directly in ELISA systems instead of indirectly via functional assays.[13] Even intracellular detection of cytokines can now be performed by flow cytometry, which allows the determination of "cytokine profiles" of lymphocyte subpopulations, such as T-helper 1 (IL2, IFNγ) and T-helper 2 (IL4, IL5, IL10), or T-naive (IL2) and T-memory (IFNγ) profiles.[7,34,50] Furthermore, many cytokines are now available in recombinant forms, allowing more precise *in vitro* functional studies for specific research purposes.

These technical improvements have greatly facilitated studies on the lymphocyte system, and contributed to better interpretation of the data obtained in these studies.

FORMER PITFALLS IN THE INTERPRETATION OF PEDIATRIC IMMUNOPHENOTYPING DATA

In the past, restrictions in technical possibilities have impeded the opportunities to test the interpretation of data from pediatric immunological studies. A clear example is the assumption that immature "thymocyte-like" lymphocyte precursors are present in the neonatal circulation, as suggested by studies on the distribution of blood lymphocyte subpopulations in the 1980's.[15,18,19,31-33,37,44] E-rosetting lymphocytes (i.e. lymphocytes binding to sheep erythrocytes) were considered to be equivalent to T-lymphocytes. Consequently, cord blood cells with a low affinity E-rosette receptor causing lower relative counts of E-rosetting lymphocytes as compared to adult cells were initially assumed to be T-cell precursors.[37,43] This idea of immature "thymocyte-like" precursors in the neonatal circulation was further strengthened by the finding of CD8+/CD3- cells[32] and CD4+/CD3- cells,[15,18] as well as CD1+ cells,[15,18,19,31,33,44] CD38+ cells,[15,18,19,33] and CD71+ cells[18,44] in cord blood MNC fractions. CD8+/CD3- and CD4+/CD3- cells were assumed to be thymocytes in a specific developmental stage, whereas CD1, CD38, and CD71 were known to be expressed by thymocytes. Also, the sum of CD4+ and CD8+ lymphocytes often exceeded the CD3+ lymphocyte count, which suggested that an appreciable number of CD4+/CD8+ "double-positive" thymocytes was present.[15,18,19,31-33,44]

Recent studies using double and triple immunostaining have revealed that no such immature "thymocyte-like" lymphocyte precursors exist in the neonatal circulation. CD8+/CD3- lymphocytes (Figure 1b) are in fact CD8+ natural killer (NK) cells (Figure 1c), which also have a low affinity E-rosette receptor.[3,38,39] The presence of relatively high numbers of CD8+ NK-cells explains the difference between the sum of CD4+ (= CD4+/CD3+) and CD8+ (=CD8+/CD3+ + CD8+/CD3-) lymphocytes and the number of CD3+ lymphocytes. Furthermore, "double-positive" CD4+/CD8+ lymphocytes were never found in significant numbers in cord blood with double immunostaining studies (Figure 1d).[3,38,39] CD4+/CD3- cells

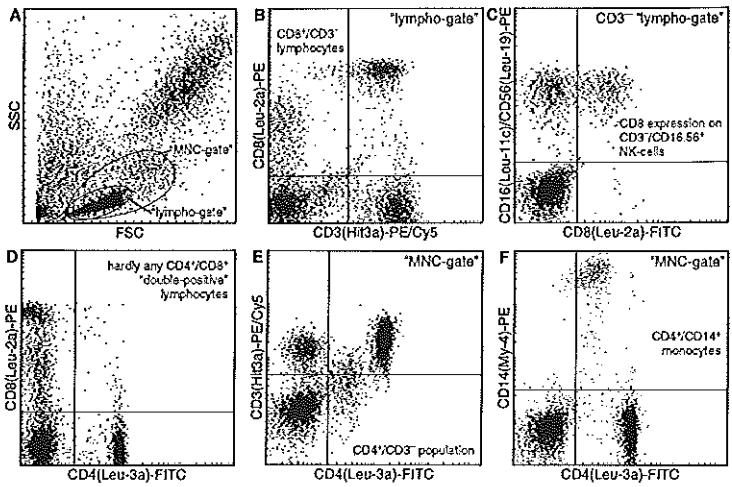

Figure 1. In neonatal cord blood, the difference between the sum of CD4+ and CD8+ cells and total CD3+ cells is caused by CD8+/CD3- NK-cells and by small CD4+/CD3- monocytes within the "lympho-gate". *A*, FSC versus SSC dot plot with "lympho-gate" and "MNC-gate"; the "MNC-gate" contains lymphocytes as well as monocytes. *B*, Relatively high frequencies of CD8+/CD3- lymphocytes are found. *C*, CD8 expression is found on CD3-/CD16.CD56+ NK-cells. *D*, Hardly any CD4+/CD8+ "double-positive" lymphocytes are present. *E*, A population of CD4+/CD3- cells is present within the "MNC-gate"; this population is partly included in the "lympho-gate" if the gate is set too wide. *F*, The CD4+/CD3- cells are CD4+/CD14+ monocytes.

(Figure 1e) are in fact small CD14+ monocytes with the same size as lymphocytes, thereby "contaminating" the "lympho-gate" (Figure 1f). The CD1+ lymphocytes were found to be CD1c+ B-lymphocytes (=CD1c+/CD19+), which occur at relatively high frequencies in cord blood (Figure 3).[11] CD71+ cells can be found in large numbers in cord blood, but most of them are not CD71+ lymphocytes, but CD71+/GlycophorinA(GpA)+/CD45- normoblasts (Figure 4). These normoblasts are mainly located within the "lympho-gate", because their size and surface irregularity are similar to those of lymphocytes, resulting in similar scatter characteristics. The presence of these normoblasts within the "lympho-gate" leads to underestimation of the size of lymphocyte subpopulations.[10] This can interfere with accurate prenatal or early neonatal diagnosis of possible congenital immunodeficiency.

CURRENT VIEWS

Because neonates and infants are more susceptible to infections, it is likely that their lymphocytes are functionally immature in comparison with adults. However, these lymphocytes are not "thymocyte-like", as was previously thought. Recent studies have shown that CD45RA+ "naive" T-cells occur at high relative frequencies in neonates and infants, whereas CD45RO+ "memory" T-cells occur

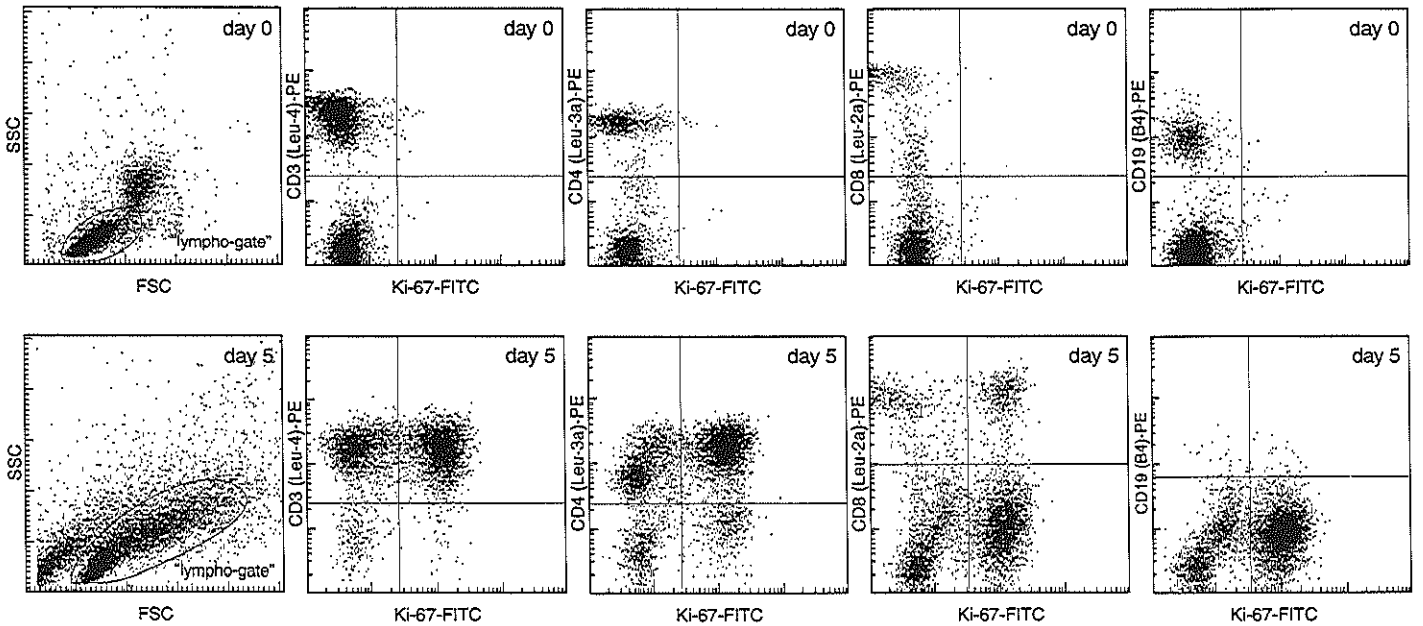

Figure 2. Evaluation of *in vitro* lymphocyte proliferation of various lymphocyte subpopulations by staining for the nuclear Ki67 proliferation antigen. Blood MNC were stimulated with *Staphylococcus aureus* protein A and evaluated for the expression of Ki67 by CD3+ T-lymphocytes, CD4+ T-lymphocytes, CD8+ T-lymphocytes, and CD19+ B-lymphocytes at day 0 (upper part) and at day 5 (lower part) of culture. This figure illustrates that *Staphylococcus aureus* protein A especially stimulates CD4+ T-lymphocytes, whereas CD8+ T-lymphocytes proliferate at much lower rates and CD19+ B-lymphocytes are virtually absent on day 5 of culture.

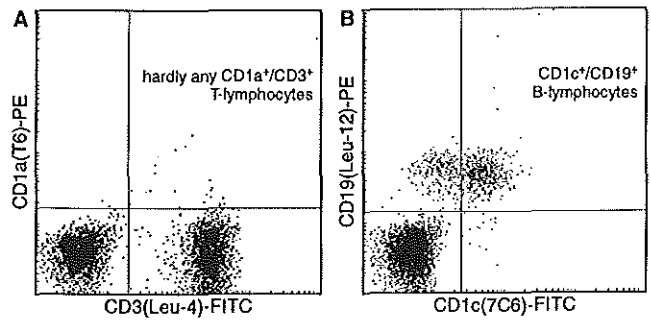

Figure 3. CD1+ cells in neonatal cord blood are B-lymphocytes, not T-lymphocytes. *A*, CD3+ T-lymphocytes in neonatal cord blood hardly express the CD1a antigen (T6, Coulter, Hialeah FL). *B*, CD1c expression (7C6, dr. W. Knapp and dr. O. Majdic, Vienna, Austria) is found on approximately 87% of CD19+ B-lymphocytes (median of 15 neonatal cord blood samples).

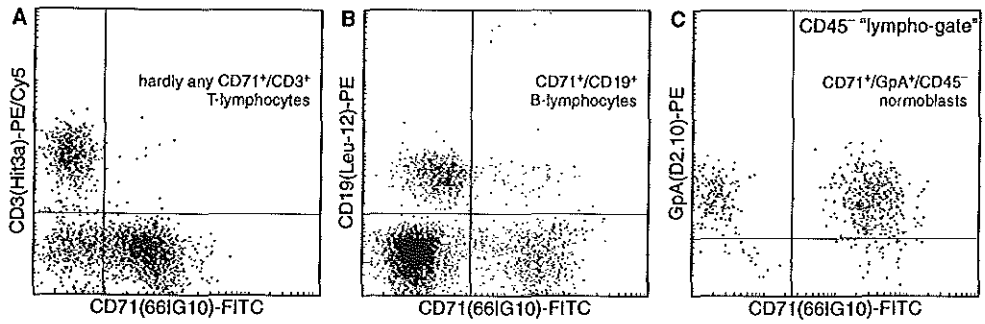

Figure 4. Most CD71+ cells within the "lympho-gate" of neonatal cord blood samples are normoblasts. *A*, CD3+ T-lymphocytes hardly express the transferrin receptor CD71. *B*, Only a small subpopulation of CD19+ B-lymphocytes in neonatal cord blood expresses CD71 (median 7% in 15 neonatal cord blood samples). *C*, Most (50 to 98% in 15 neonatal cord blood samples) of CD71+ cells within the "lympho-gate" of neonatal cord blood are CD71+/GpA+/CD45- normoblasts.

at lower relative frequencies.[1,3,14,21,24] Functional differences fit in with this CD45RA/CD45RO distribution: decreased proliferation of T-cells after stimulation with McAb, decreased T-helper function, and the cytokine profile all belong to the "naive" CD45RA+ phenotype.[2,4,12,17,27,28,42,45,47-50]

Tables 1 and 2 summarize recent literature data about the relative frequencies of lymphocyte subpopulations in neonates and infants as compared to adults.[1,3,5,6,8,9,11,14,16,21,23-26,36,39,40] It is important to realize that relative frequencies of lymphocyte subpopulations may be smaller than in adults, but that absolute numbers are higher due to the increased number of lymphocytes in neonates and infants.[8] Also, unidentified normoblasts in the neonatal circulation may have influenced the outcome of the studies performed in cord blood.[10]

TABLE 1. Literature data on the relative frequencies of T-lymphocyte subpopulations in the first years of life as compared to adults.

	Pan-T-cell markers	Helper/ suppressor markers		Activation markers				Naive/memory markers			TCR molecule	Other
	CD2 and CD3	CD4 and CD8	4/8 ratio	CD25	HLA-DR	CD69	CD40L	CD45RA	CD45RO	CD29	TCRγδ	CD38
Neonatal cord blood	↓	contra-dictory results	↓	=	↓	=	↓	↑↑	↓	↓	↓	↑↑
Infant blood	↗	contra-dictory results	↘	=		=		↘	↗	↗	↗	↘
Literature	Refs. 1, 2, 4, 24, 26, 28, 30, 43-46	Refs. 1, 2, 4, 24, 26, 28-30, 43-46		Refs. 1, 8, 24, 46, 47				Refs. 2, 24, 28, 29, 44, 48			Refs. 24, 44	Refs. 24, 28

↑: higher compared to adults, ↑↑: much higher compared to adults, ↓: lower compared to adults, =: similar to adults, ↗: increase during infancy, ↘: decrease during infancy; TCR = T-cell receptor.

TABLE 2. Literature data on the relative frequencies of B-lymphocyte and NK-cell subpopulations in the first years of life as compared to adults.

	Pan-B-cell markers	"T-cell associated" markers		$F_c\gamma$ receptor	NK-cell markers	
	CD19 and CD20	CD5	CD1c	CD16	CD56	CD57
Neonatal cord blood	↓	↓	↓	↓	↓	absent
Infant blood	↗↘	↘		↘	↗	↗
Literature	Refs. 1, 2, 4, 24, 27, 28, 44-46	Refs. 2, 27, 44		Refs. 1, 2, 24, 28, 46, 50	Refs. 1, 2, 4, 24, 44, 46, 50	

↑: higher compared to adults, ↓: lower compared to adults
↗: increase during infancy, ↘: decrease during infancy, ↗↘: first increase, than decrease during infancy

Table 3 summarizes the current knowledge about lymphocyte function.[2,4,12,17,27,28,41,42,45-50] The information in this table indicates that T-lymphocytes, B-lymphocytes, and NK-cells in neonates and infants are indeed functionally immature.

The age-related changes summarized in Tables 1-3 clearly underline the importance of using age-matched controls and reference values when analyzing the pediatric lymphocyte system.

TABLE 3. Literature data on lymphocyte function in the first year of life.

Lymphocyte subsets and functions	Literature
T-lymphocytes	
- Helper function of T-lymphocytes for immunoglobulin (Ig) production by B-lymphocytes is lower in neonatal cord blood than in adult blood; adult B-lymphocytes produce less Ig when helped by neonatal T-lymphocytes instead of by adult T-lymphocytes or their respective culture supernatants. Neonatal T-lymphocytes cannot induce neonatal B-lymphocytes to produce IgG, whereas adult T-lymphocytes can.	Refs. 36, 37, 49
- Proliferation after stimulation with mitogens is similar in neonatal cord blood and adult blood. Proliferation is lower after stimulation with McAb in neonatal cord blood, which can be overcome by adding another stimulus.	Refs. 6, 31-35
- The production of IL2 is similar in neonatal cord blood and adult blood; the production of IFNγ is lower in neonatal cord blood; the production of IL4 is virtually absent in neonatal cord blood.	Refs. 6, 31-35, 38-41
B-lymphocytes	
- Ig production is restricted to the IgM isotype during the first month of life; IgG and IgA appear later.	Ref. 36
- Ig production after Epstein-Barr virus stimulation of neonatal cord blood cells *in vitro* is restricted to the IgM isotype as well.	Ref. 36
NK-cells	
- NK cytotoxicity is lower in neonatal cord blood than in adult blood in some but not all infants; limited information is available.	Ref. 50

FUTURE PERSPECTIVES

Although nowadays much is known about the development of lymphocyte phenotype and function in healthy neonates and infants, many questions remain. Extensive knowledge of the normal development of the immune system during the first years of life and the availability of reliable tests with proper reference values for neonates and infants is of utmost importance to optimize our care for children with immune disorders like HIV-infection and congenital immunodeficiency disease, and for children after bone marrow transplantation, or children with cancer during and after intensive courses of chemotherapy.

The development of microassays for immunophenotyping as well as for functional testing of lymphocytes will make it possible to undertake more extensive studies in neonates and young infants, and to obtain adequate reference values. Precise identification and enumeration of normoblasts within the "lympho-gate" will

enhance the value of results obtained from neonatal cord blood. Preferably, longitudinal studies should be performed to assess the course of lymphocyte development during the first years of life in relation to factors like nutrition, infections, and vaccinations. This will lead to a better understanding of the interaction between the pediatric immune system and the pathogens with which it is challenged.

ACKNOWLEDGEMENTS We are grateful to Prof. dr. R. Benner and Dr. H. Hooijkaas for their continuous support and to mr. T.M. van Os for preparation of the figures.

REFERENCES

1 Aldhous MC, Raab GM, Doherty KV, Mok JYQ, Bird AG, Froebel KS. Age-related ranges of memory, activation, and cytotoxic markers on CD4 and CD8 cells in children. *J Clin Immunol* 1994;14:289-298.

2 Andersson U, Bird G, Britton S. Cellular mechanisms of restricted immunoglobulin formation in the human neonate. *Eur J Immunol* 1980;10:888-894.

3 Beck R, Lam-Po-Tang PRL. Comparison of cord blood and adult blood lymphocyte normal ranges: A possible explanation for decreased severity of graft versus host disease after cord blood transplantation. *Immunol Cell Biol* 1994;72:440-444.

4 Bertotto A, Gerli R, Lanfrancone L, Crupi S, Arcangeli C, Cernetti C, Spinozzi F, Rambotti P. Activation of cord T lymphocytes. II. Cellular and molecular analysis of the defective response induced by anti-CD3 monoclonal antibody. *Cell Immunol* 1990;127:247-259.

5 Brugnoni D, Airò P, Graf D, Marconi M, Lebowitz M, Plebani A, Giliani S, Malacarne F, Cattaneo R, Ugazio AG, et al. Ineffective expression of CD40 ligand on cord blood T cells may contribute to poor immunoglobulin production in the newborn. *Eur J Immunol* 1994;24:1919-1924.

6 Carver JD, Pimentel B, Wiener DA, Lowell NE, Barness LA. Infant feeding effects on flow cytometric analysis of blood. *J Clin Lab Analysis* 1991;5:54-56.

7 Clerici M, De Palma L, Roilides E, Baker R, Shearer GM. Analysis of T helper and antigen-presenting cell functions in cord blood and peripheral blood leukocytes from healthy children of different ages. *J Clin Invest* 1993;91:2829-2836.

8 Comans-Bitter WM, De Groot R, Van den Beemd R, Neijens HJ, Hop WCJ, Groeneveld K, et al. Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. *J Pediatr* 1997;130:388-393.

9 Denny T, Yogev R, Gelman R, Skuza C, Oleske J, Chadwick E, Cheng S, Connor E. Lymphocyte subsets in healthy children during the first 5 years of life. *J Am Med Assoc* 1992;267:1484-1488.

10 De Vries E, De Bruin-Versteeg S, Comans-Bitter WM, De Groot R, Boerma GJM, Lotgering FK, Van Dongen JJM. Correction for erythroid cell contamination in microassay for immunophenotyping of neonatal lymphocytes. *Arch Dis Child (in press).*

11 Durandy A, Thuillier L, Forveille M, Fischer A. Phenotypic and functional characteristics of human newborns' B lymphocytes. *J Immunol* 1990;144:60-65.

12 Ehlers S, Smith KA. Differentiation of T cell lymphokine gene expression: the in vitro acquisition of T cell memory. *J Exp Med* 1991;173:25-36.

13 English KB, Burchett SK, English JD, Ammann AJ, Wara DW, Wilson CB. Production of lymphotoxin and tumor necrosis factor by human neonatal mononuclear cells. *Pediatr Res* 1988;24:717-722.

14 Erkeller-Yuksel FM, Deneys V, Yuksel B, Hannet I, Hulstaert F, Hamilton C, et al. Age-related changes in human blood lymphocyte subpopulations. *J Pediatr* 1991;120:216-222.

15 Foa R, Giubellino MC, Fierro MT, Lusso P, Ferrando ML. Immature T lymphocytes in human cord blood identified by monoclonal antibodies: a model for the study of the diffentiation pathway of T cells in humans. *Cell Immunol* 1984;89:194-201.

16 Fuleihan R, Ahern D, Geha RS. Decreased expression of the ligand for CD40 in newborn lymphocytes. *Eur J Immunol* 1994;24:1925-1928.

17 Gerli R, Bertotto A, Crupi S, Arcangeli C, Marinelli I, Spinozzi F, Cernetti C, Angelella P, Rambotti

P. Activation of cord T lymphocytes. I. Evidence for a defective T cell mitogenesis induced through the CD2 molecule. *J Immunol* 1989;142:2583-2589.
18 Gerli R, Rambotti P, Cernetti C, Velardi A, Spinozzi F, Tabilio A, Martelli MF, Grignani F, Davis S. A mature thymocyte-like phenotypic pattern on human cord circulating T-lymphoid cells. *J Clin Immunol* 1984;4:461-468.
19 Griffiths-Chu S, Patterson JAK, Berger CL, Edelson RL, Chu AC. Characterization of immature T cell subpopulations in neonatal blood. *Blood* 1984;64:296-300.
20 Groeneveld K, Te Marvelde JG, Van den Beemd R, Slijkoord EJ, Hooijkaas H, Van Dongen JJM. Detection of intracellular antigens. In: Van Dongen JJM, Groeneveld K, Adriaansen HJ, Hooijkaas H (eds) *Immunophenotyping in diagnostics: indications, execution and interpretation.* Dept. of Immunology, Erasmus University Rotterdam, Rotterdam,1994, pp 63-73.
21 Heldrup J, Kalm O, Prellner K. Blood T and B lymphocyte subpopulations in healthy infants and children. *Acta Paediatr* 1992;81:125-132.
22 Iatropoulos MJ, Williams GM. Proliferation markers. *Exp Toxicol Pathol* 1996;48:175-181.
23 Ibegbu C, Spira TJ, Nesheim S, Mendez H, Lee F, Polliotti B, Caba J, Nahmias A. Subpopulations of T and B cells in perinatally HIV-infected and noninfected age-matched children compared with those in adults. *Clin Immunol Immunop* 1994;71:27-32.
24 Kotylo PK, Baenzinger JC, Yoder MC, Engle WA, Bolinger CD. Rapid analysis of lymphocyte subsets in cord blood. *AJCP* 1990;93:263-266.
25 Kotylo PK, Fineberg NS, Freeman KS, Redmond NL, Charland C. Reference ranges for lymphocyte subsets in pediatric patients. *Hematopathol* 1992;100:111-115.
26 Kutvirt SG, Lewis SL, Simon TL. Lymphocyte phenotypes in infants are altered by separation of blood on density gradients. *Brit J Biomed Science* 1993;50:321-328.
27 Lewis DB, Larsen A, Wilson CB. Reduced interferon-gamma mRNA levels in human neonates. *J Exp Med* 1986;163:1018-1023.
28 Lewis DB, Yu CC, Meyer J, English BK, Kahn SJ, Wilson CB. Cellular and molecular mechanisms for reduced interleukin 4 and interferon-gamma production by neonatal T cells. *J Clin Invest* 1991;87:194-202.
29 Lewis DB, Wilson CB. Developmental immunology and role of host defences in neonatal susceptibility to infection. In: Remington JS, Klein JO (eds) *Infectious diseases of the fetus and newborn infant.* Saunders, Philadelphia, 1995, pp 20-98.
30 Loken MR, Brosnan JM, Bach BA, Ault KA. Establishing optimal lymphocyte gates for immunophenotyping by flow cytometry. *Cytometry* 1990;11:453-459.
31 Lucivero G, Dell'Osso A, Iannone A, Selvaggi L, Antonaci S, Bettocchi S, Bonomo L. Phenotypic immaturity of T and B lymphocytes in cord blood of full-term normal neonates. Analysis of cell surface markers by using conventional techniques and monoclonal antibodies. *Biol Neonate* 1983;44:303-308.
32 Maccario R, Nespoli L, Mingrat G, Vitiello A, Ugazio AG, Burgio GR. Lymphocyte subpopulations in the neonate: identification of an immature subset of OKT8-positive, OKT3-negative cells. *J Immunol* 1983;130:1129-1131.
33 Moretta A, Valtorta A, Chirico G, Chiara A, Bozzola M, DeAmici M, Maccario R. Lymphocyte subpopulations in preterm infants: high percentage of cells expressing p55 chain of interleukin-2 receptor. *Biol Neonate* 1991;59:213-218.
34 North ME, Ivory K, Funauchi M, Webster AD, Lane AC, Farrant J. Intracellular cytokine production by human CD4+ and CD8+ T cells from normal and immunodeficient donors using directly conjugated anti-cytokine antibodies and three-colour flow cytometry. *Clin Exp Immunol* 1996;105:517-522.
35 Oski F. The erythrocyte and its disorders. In: Nathan D, Oski F (eds) *Hematology of infancy and childhood.* 4th ed. Saunders, Philadelphia, 1993, pp 18-43.
36 Pilarski LM, Yacyshyn BR, Jensen GS, Pruski E, Pabst HF. beta1 integrin (CD29) expression on human postnatal T cell subsets defined by selective CD45 isoform expression. *J Immunol* 1991;147:830-837.
37 Porta FA, Maccario R, Ferrari FA, Alberini CM, Montagna D, DeAmici M, Gianelli A, Ugazio AG. Lymphocyte subpopulations in the neonate: high percentage of ANAE+ cells with low avidity for sheep erythrocytes. *Thymus* 1985;7:263-269.
38 Reason DC, Ebisawa M, Saito H, Nagakura T, Iikura Y. Human cord blood lymphocytes do not simultaneously express CD4 and CD8 cell surface markers. *Biol Neonate* 1990;58:87-90.
39 Remy N, Oberreit M, Thoenes G, Wahn U. Lymphocyte subsets in whole blood and isolated mononuclear leucocytes of healthy infants and children. *Eur J Pediatr* 1991;150:230-233.

40 Samelson R, Larkey DM, Amankwah KS, McConnachie P. Effect of labor on lymphocyte subsets in full-term neonates. *Am J Reprod Immunol* 1992;28:71-73.
41 Sancho L, DeLaHera A, Casas J, Vaquer S, Martínez-A C, Alvarez-Mon M. Two different maturational stages of natural killer lymphocytes in human newborn infants. *J Pediatr* 1991;119:446-454.
42 Seki H, Taga K, Matsuda A, Uwadana N, Hasui M, Miyawaki T, Taniguchi N. Phenotypic and functional characteristics of active suppressor cells against IFN-gamma production in PHA-stimulated cord blood lymphocytes. *J Immunol* 1986;137:3158-3161.
43 Smith MA, Evans J, Steel CM. Age-related variation in proportion of circulating T cells. *Lancet* 1974;2(7886):922-924.
44 Solinger AM . Immature T lymphocytes in human neonatal blood. *Cell Immunol* 1985;92:115-122.
45 Splawski JB, Jelinek DF, Lipsky PE. Delineation of the functional capacity of human neonatal lymphocytes. *J Clin Invest* 1991;87:545-553.
46 Splawski JB, Lipsky PE. Cytokine regulation of immunoglobulin secretion by neonatal lymphocytes. *J Clin Invest* 1991;88:967-977.
47 Taylor S, Bryson YJ. Impaired production of gamma-interferon by newborn cells in vitro is due to a functionally immature macrophage. *J Immunol* 1984;134:1493-1497.
48 Wakasugi N, Virelizier J. Defective IFN-gamma production in the human neonate. I. Dysregulation rather than intrinsic abnormality. *J Immunol* 1985;134:167-171.
49 Wakasugi N, Virelizier J, Arenzana-Seisdedos F, Rothhut B, Mencia Huerta J, Russo-Marie F, Fiers W. Defective IFN-gamma production in the human neonate. II. role of increased sensitivity to the suppressive effects of prostaglandin. *Eur J Immunol* 1985;134:172-176.
50 Wilson CB, Westall J, Johnston L, Lewis DB, Dower SK, Alpert AR. Decreased production of interferon-gamma by human neonatal cells. Intrinsic and regulatory deficiencies. *J Clin Invest* 1986;77:860-867.

CHAPTER 5

NEONATAL BLOOD LYMPHOCYTE SUBPOPULATIONS: A DIFFERENT PERSPECTIVE WHEN USING ABSOLUTE COUNTS

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ABSTRACT

We compared the absolute counts of lymphocyte subpopulations in 5 preterm neonates, 15 term neonates, and 9 adults using the whole lysed blood technique with 15 different triple immunostainings. To obtain accurate absolute lymphocyte counts in neonatal cord blood samples, the flow cytometric "lympho-gate" was corrected for the erythroid cell contamination by normoblasts and unlysed erythrocytes.

In contrast to earlier studies where relative frequencies were reported, we found that the major difference between neonatal and adult lymphocyte subpopulations concerned the much larger pool of naive "untriggered" cells in neonates, standby for participation in primary immune responses. In some preterm neonates this pool of "naive" untriggered cells was smaller than in term neonates, which might explain their higher susceptibility to infections, due to a slower or less efficient primary immune response as compared to term neonates.

INTRODUCTION

Neonates have an increased susceptibility to infections due to a functional immaturity of their immune system[1] which has not yet been challenged by antigens. Detailed flow cytometric analysis of blood lymphocyte subpopulations in preterm and term neonates in comparison with data obtained in adults can contribute to a better understanding of this functional immaturity. This is most easily performed in neonatal cord blood which is available in large quantities without the need for venipuncture of the child itself. Neonates have higher blood lymphocyte counts than adults.[2,3] Therefore, the actual differences in size of lymphocyte subpopulations between neonates and adults are better reflected by comparison of their absolute counts than by comparison of their relative frequencies.[3]

However, it is difficult to obtain accurate absolute counts of lymphocyte subpopulations by flow cytometric immunophenotyping of neonatal cord blood. This inaccuracy is caused by erythroid cell contamination of the so-called "lympho-gate". Blood lymphocytes have a relatively homogeneous size and surface membrane morphology which give characteristic forward scatter (FSC) and side scatter (SSC) patterns in flow cytometric immunophenotyping that can be used for defining the "lympho-gate" (Figure 1a).[4] Normoblasts are erythroid precursors, which can be present in considerable quantities in neonatal cord blood.[2,5] They have comparable size and scatter characteristics as lymphocytes, and are mainly located within this "lympho-gate". Furthermore, neonatal erythrocytes are relatively resistant to lysis,[2] and are sometimes large enough to be located within the "lympho-gate" as well.[5] This erythroid contamination of the "lympho-gate" disturbs the determination of lymphocyte subpopulations.[5] The transferrin receptor CD71 is expressed on erythroid precursors, and glycophorin A (GpA) is expressed on all erythroid cells. CD45 is expressed on all leukocytes. Therefore, we used the

CD71/GpA/CD45 triple immunostaining for recognition of the erythroid cell contamination of the "lympho-gate"; this prevents underestimation of the relative frequencies of the various lymphocyte subpopulations and allows accurate calculation of absolute counts.[5]

We studied the absolute counts of lymphocyte subpopulations in fifteen term neonatal cord blood samples and five preterm neonatal cord blood samples, and compared the results with data obtained in nine adult peripheral blood samples. We especially studied markers that have been reported to be present in different relative frequencies on neonatal as compared to adult lymphocytes,[6,7] to see whether comparing the actual numbers of these subpopulations would yield a different insight in the neonatal immune system.

MATERIALS AND METHODS

Cell samples

Neonatal cord blood was collected by venipuncture immediately after clamping of the cord from fifteen healthy term infants born after an uncomplicated pregnancy and delivery (gestational age 37-41 weeks), and from five preterm infants (gestational age of 29, 30, 33, 34 and 35 weeks). All preterm infants showed an uncomplicated course in the neonatal period, except the child born at 29 weeks, who was ventilated for presumed bacterial infection. The blood was kept at room temperature until immunostaining was performed within 12 hours after sampling. Adult peripheral blood was drawn by venipuncture from nine healthy adult volunteers, aged 20 to 40 years. Ethylene-diamino-tetra-acetate was used as anti-coagulant. Informed consent was obtained according to the guidelines of the Medical Ethics Committee of the Erasmus University Rotterdam / University Hospital Rotterdam.

Flow cytometric analysis

The monoclonal antibodies (McAb; listed in Table 1) were conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), or the duochrome PE-Cyanin5 (PE-Cy5). The same set of fifteen triple immunostainings was used in all samples to measure T-lymphocytes (CD3+), B-lymphocytes (CD19+), NK-cells (CD16+ and/or CD56+ / CD3-), and their various subpopulations.

The lysed whole blood technique was used for all stainings as described previously.[5] Briefly, 20 µl of blood was incubated with three optimally titrated McAb for 10 minutes at room temperature. Erythrocytes were lysed with Lysing Solution (Becton Dickinson, San Jose, CA). The CD71/GpA/CD45 triple immunostaining was included to identify normoblasts and unlysed erythrocytes within the "lympho-gate". Samples were analyzed with a FACScan flow cytometer, using FACScan Research software (Becton Dickinson).

TABLE 1. Immunostainings used in the study.

FITC-conjugated McAb		PE-conjugated McAb		PE-Cy5-conjugated McAb		Recognized lymphocyte subpopulation
Marker	McAb clone*	Marker	McaAb clone	Marker	McAb clone	
IgG1	IgG1 (BD)	IgG2	IgG2 (BD)	IgG1	679.1Mc7 (IT)	Isotype controls
CD15	CLB-gran2 (CLB)	CD14	My-4 (CT)	CD45	HI30 (PH)	"Lympho-gate" contents control
CD71	66IG10 (MS)	GpA	D2.10 (PH)	CD45	HI30 (PH)	Correction for "erythroid contamination" of "lympho-gate"
CD5	Leu-1 (BD)	CD19	Leu-12 (BD)	CD3	Hitt3a (PH)	CD5+/CD19+ and CD5-/CD19+ subpopulations
CD1c	7C6 (KM)	CD19	Leu-12 (BD)	CD3	Hitt3a (PH)	CD1c+/CD19+ and CD1c-/CD19+ subpopulations
CD71	66IG10 (MS)	CD19	Leu-12 (BD)	CD3	Hitt3a (PH)	Control of CD71 expression on lymphocytes
CD4	Leu-3a (BD)	CD8	Leu-2a (BD)	CD3	Hitt3a (PH)	CD4+/CD3+ and CD8+/CD3+ subpopulations
CD4	Leu-3a (BD)	CD45RA	2H4 (CT)	CD3	Hitt3a (PH)	CD4+/CD3+ and CD45RA+/CD3+ subpopulation
CD8	Leu-2a (BD)	CD45RA	2H4 (CCT)	CD3	Hitt3a (PH)	CD8+/CD3+ and CD45RA+/CD3+ subpopulation
CD4	Leu-3a (BD)	CD45RO	UCHL-1 (DK)	CD3	Hitt3a (PH)	CD4+/CD3+ and CD45RO+/CD3+ subpopulation
CD8	Leu-2a (BD)	CD45RO	UCHL-1 (DK)	CD3	Hitt3a (PH)	CD8+/CD3+ and CD45RO+/CD3+ subpopulation
CD45RO	UCHL-1 (DK)	CD45RA	2H4 (CT)	CD3	Hitt3a (PH)	CD45RA+/CD3+ and CD45RO+/CD3+ subpopulations
CD8	Leu-2a (BD)	CD16/CD56	Leu-11c/ Leu-119 (BD)	CD3	Hitt3a (PH)	CD8+ and CD8- NK-cells (CD16 and/or CD56+ and CD3-)
CD45RO	UCHL-1 (DK)	CD16/CD56	Leu-11c/ Leu-19 (BD)	CD3	Hitt3a (PH)	CD45RA+/CD3+ and CD45RO+/CD3+ subpopulations
CD45RA	Leu-18 (BD)	CD16/CD56	Leu-11c/ Leu-19 (BD)	CD3	Hitt3a (PH)	CD45RA+/CD3+ and CD45RO+/CD3+ subpopulations

* Companies: BD = Becton Dickinson, San Jose, CA; CLB = Central Laboratory of the Blood Transfusion Service, Amsterdam, the Netherlands; MS = Monosan Sanbio, Uden, the Netherlands; IT = Immunotech, Marseille, France; KM = Dr. W. Knapp and dr. O. Madjic, Vienna, Austria; DK = Dakopatts, Glostrup, Denmark; CT = Coulter Clone, Hialeah, FL; PH = Pharmingen, San Diego, CA.

TABLE 2. Relative frequencies of T- and B-lymphocyte and NK-cell (sub)populations in neonates and adults.[a]

	Preterm neonates (n=5)	Term neonates (n=15)	p-value[b] preterm vs term	Adults (n=9)	p-value[b] term vs adult
CD3+ T-lymphocytes	**52 (44-65)**	**64 (46-76)**	**NS**	**72 (60-83)**	**0.023**
CD4+ subpopulation per CD3+	75 (68-88)	73 (62-83)	NS	58 (52-69)	<0.001
CD8+ subpopulation per CD3+	20 (13-26)	27 (16-35)	NS	33 (27-46)	0.002
CD4/CD8 ratio	3.8 (2.6-6.7)	2.7 (1.8-5.2)	NS	2.0 (1.2-2.7)	0.001
CD45RA+ subpopulation per CD3+	54 (38-90)	72 (44-95)	NS	60 (46-71)	NS
CD45RO+ subpopulation per CD3+	28 (11-41)	21 (10-76)	NS	56 (48-71)	<0.001
CD19+ B-lymphocytes	**17 (8-31)**	**14 (9-21)**	**NS**	**12 (7-18)**	**NS**
CD5+ subpopulation per CD19+	38 (31-83)	42 (25-62)	NS	23 (13-30)	<0.001
CD1c+ subpopulation per CD19+	75 (59-93)	86 (65-92)	0.041	25 (21-29)	<0.001
CD71+ subpopulation per CD19+	14 (8-45)	7 (2-20)	NS	8 (3-27)	NS
CD16+/56+/CD3- NK-cells	**17 (8-31)**	**22 (9-33)**	**NS**	**14 (4-18)**	**0.016**
CD8+ subpopulation per CD16+/56+/CD3-	22 (19-38)	37 (24-63)	0.017	29 (11-44)	NS

TABLE 3. Absolute counts of total lymphocytes and T- and B-lymphocyte and NK-cell (sub)populations in neonates and adults.[c]

	Preterm neonates (n=5)	Term neonates (n=15)	p-value[b] preterm vs term	Adults (n=9)	p-value[b] term vs adult
Total lymphocytes	**4.5 (1.8-6.4)**	**4.9 (3.5-6.3)**	**NS**	**2.1 (1.1-2.4)**	**<0.001**
CD3+ T-lymphocytes	**2.3 (1.0-3.3)**	**3.3 (1.9-4.4)**	**NS**	**1.5 (0.7-1.8)**	**<0.001**
CD4+/CD3+ subpopulation	1.6 (0.8-2.9)	2.3 (1.4-3.5)	NS	1.0 (0.4-1.1)	<0.001
CD8+/CD3+ subpopulation	0.4 (0.2-0.6)	0.7 (0.4-1.1)	0.008	0.5 (0.3-0.8)	0.004
CD45RA+/CD3+ subpopulation	1.0 (0.4-3.0)	1.9 (1.2-3.9)	NS	0.9 (0.3-1.1)	<0.001
CD45RO+/CD3+ subpopulation	0.6 (0.3-1.2)	0.6 (0.3-2.2)	NS	0.8 (0.5-1.0)	NS
CD19+ B-lymphocytes	**0.9 (0.3-1.0)**	**0.8 (0.3-1.0)**	**NS**	**0.2 (0.1-0.4)**	**<0.001**
CD5+/CD19+ subpopulation	0.3 (0.2-0.3)	0.3 (0.1-0.4)	NS	0.1 (0.0-0.1)	<0.001
CD5-/CD19+ subpopulation	0.6 (0.1-0.7)	0.4 (0.2-0.6)	NS	0.2 (0.1-0.3)	<0.001
CD1c+/CD19+ subpopulation	0.5 (0.2-0.7)	0.6 (0.3-0.9)	NS	0.1 (0.0-0.1)	<0.001
CD1c-/CD19+ subpopulation	0.2 (0.0-0.4)	0.1 (0.0-0.3)	NS	0.2 (0.1-0.2)	NS
CD16+/56+/CD3- NK-cells	**0.7 (0.2-1.9)**	**1.2 (0.4-1.5)**	**NS**	**0.2 (0.1-0.4)**	**<0.001**
CD8+/CD16+/56+/CD3- subpopulation	0.1 (0.1-0.7)	0.4 (0.1-0.5)	NS	0.1 (0.0-0.1)	<0.001

[a] Median percentage followed by minimal and maximal percentages between parentheses.
[b] Only significant differences are listed (α=0.05); NS = not significant.
[c] Median absolute count (x 10^9/l) followed by minimal and maximal absolute count (x 10^9/l) between parentheses.

Lymphocyte count and differentiation

The total nucleated cell count was determined on an H1 Technicon hemocytometer (Bayer, Tarrytown, NY). The relative frequency of normoblasts was determined by manual differentiation of 400 nucleated cells. The total lymphocyte count was determined according to the following formula:

total nucleated cell count x (100 - % of normoblasts) x (% of lymphocytes) x 10^{-4}.

Lymphocyte subpopulations were calculated according to the following formula:

$$\frac{\text{percentage of lymphocyte subpopulation within the "lympho-gate"}}{\text{100 - (percentage of normoblasts plus unlysed erythrocytes within the "lympho-gate")}}$$

Statistics

After logarithmic transformation, the Student t-test for independent samples with either equal or inequal variance (tested by Levene's test for equality of variance) was used to evaluate the differences in the relative frequencies and absolute counts of lymphocyte subpopulations between preterm and term neonates and between term neonates and adults (α=0.05).

RESULTS

1. Technical aspects

Erythroid cell contamination

Within the "lympho-gate", we found a median of 9% (range 4-29%) GpA+/CD45- erythroid cells in term neonates and 27% (range 11-49%) in preterm neonates. Most of these erythroid cells were CD71+/GpA+/CD45- normoblasts, which were present in all neonatal samples. More than 1% unlysed erythrocytes (CD71-/GpA+/CD45-) were present in 7 out of 15 term neonatal samples and in all five preterm neonatal samples.

CD71+: erythroid cells, not lymphocytes

Most CD71+ cells (50-98%) within the "lympho-gate" of neonatal cord blood were CD71+/GpA+/CD45- normoblasts (Figure 1). The relative frequencies of CD71+/CD19+ B-lymphocytes in term neonates and adults were similar (Table 2); hardly any CD71+/CD3+ T-lymphocytes (median 0.5%) were observed in neonatal or adult blood.

Pitfalls in McAb analysis

In neonatal cord blood as well as in adult blood, CD3, CD4, and CD19 showed homogeneous expression patterns, resulting in dot plots with clear positivity and negativity (Figure 2a); the CD8 expression pattern was different on CD3+ T-lymphocytes ($CD8_{high}$) and CD3- NK-cells ($CD8_{intermediate}$) (Figure 2b). These markers are easy to analyse by flow cytometry. However, the CD45RA and

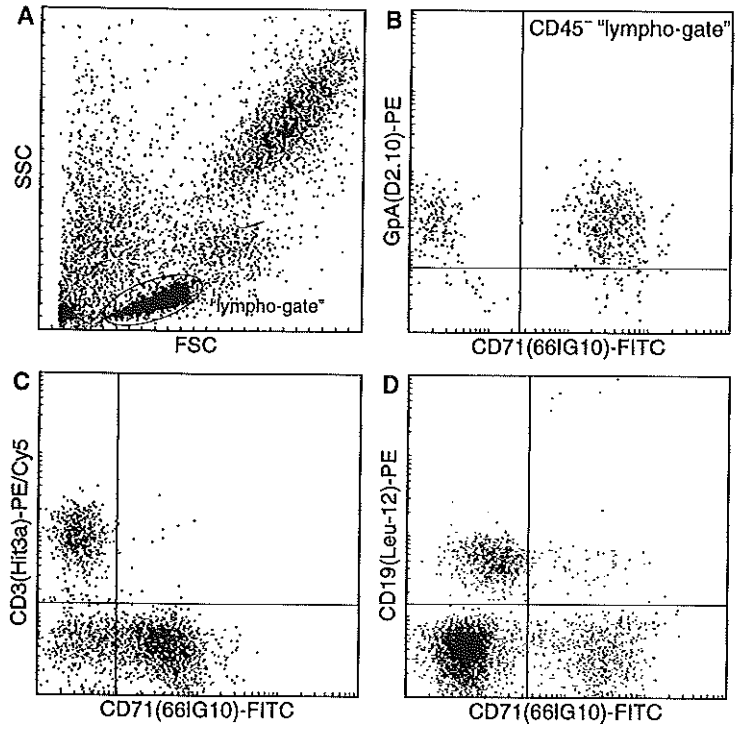

Figure 1. Most CD71-positive cells within the "lympho-gate" of neonatal cord blood samples are normoblasts. Neonatal cord blood sample. *A*, FSC versus SSC dot plot with "lympho-gate". *B*, Most CD71-positive cells within the "lympho-gate" of neonatal cord blood are CD71+/GpA+/CD45- normoblasts. *C*, CD3+ T-lymphocytes in neonatal cord blood hardly express the transferrin receptor CD71. *D*, A minor subpopulation of CD19+ B-lymphocytes in neonatal cord blood expresses CD71.

CD45RO isoforms showed heterogeneous expression patterns, ranging from negativity to high levels of expression (Figure 2c-h). This is in accordance with data from the literature.[8,9] In addition, we found a clear difference in density of expression of CD45RO, and to a lesser extent of CD45RA, between neonates and adults (Figure 2c/d); a large population of CD45RA$_{low}$/CD45RO$_{low}$ T-lymphocytes was present in the neonates, as has been described before.[9] Furthermore, the intensity of staining was influenced by the used antibody as well as by the fluorochrome conjugation (FITC or PE): PE-conjugated McAb showed a greater intensity of staining, as has been discribed before.[8] This difference was most pronounced with CD45RA and CD45RO McAb in the neonatal cord blood samples (Figure 2e-h).

2. Lymphocyte subpopulations

The main lymphocyte populations

Although the relative frequencies of T-lymphocytes were lower, B-lymphocytes similar, and NK-cells higher in term neonates as compared to adults (Table 2), the absolute counts of T- and B-lymphocytes and NK-cells were all higher in the term neonates as compared to adults, due to their higher absolute lymphocyte counts.

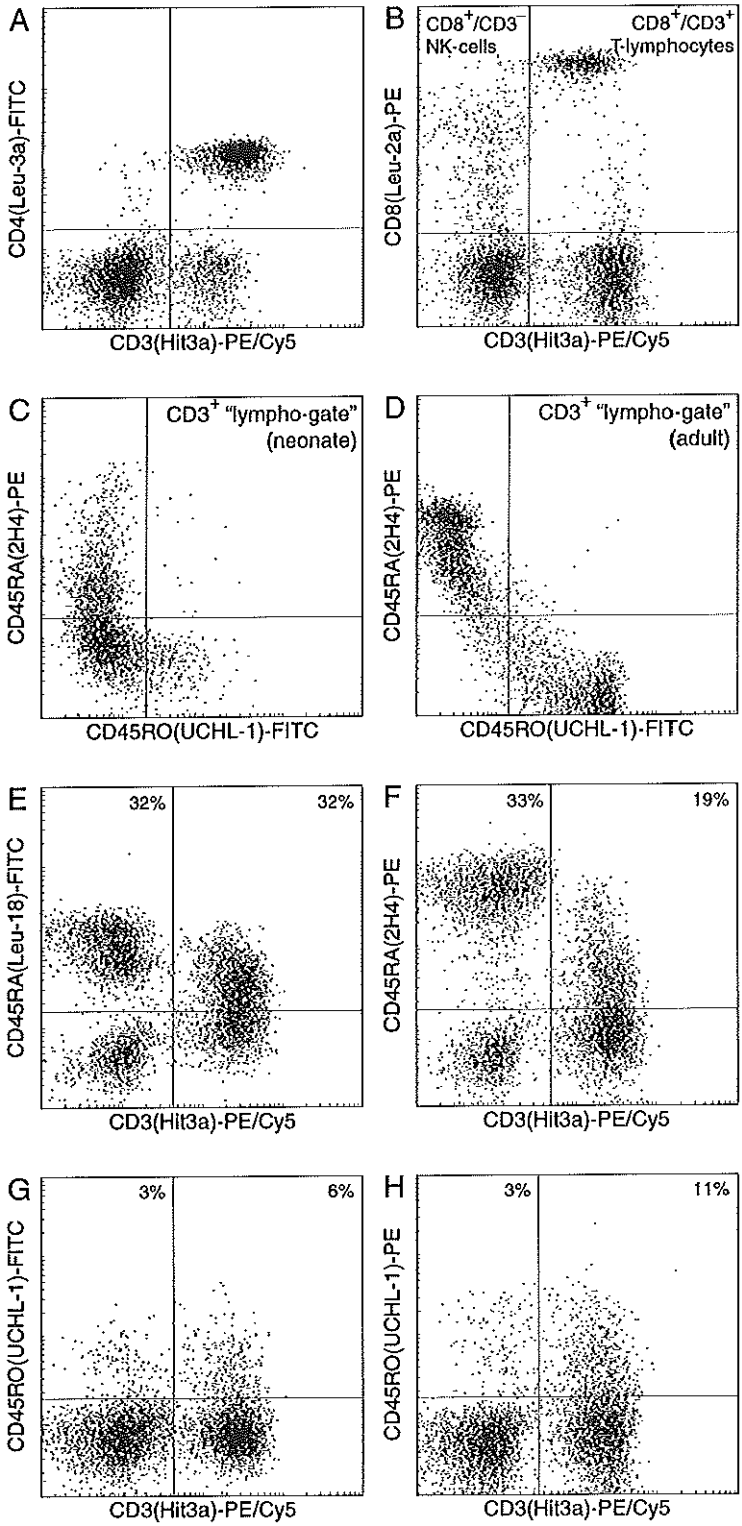

Figure 2. Pitfalls in the analysis of CD45RA and CD45RO expression by T-lymphocytes. All analyses were performed in neonatal cord blood samples (unless stated otherwise) within the flow cytometric "lympho-gate". *A*, Clear positivity and negativity in CD3 versus CD4 dot plot. *B*, Heterogeneous CD8 expression analyzed separately on T-lymphocytes (CD3+) and NK-cells (CD3-): T-lymphocytes are either CD8high or CD8negative, CD8+ NK-cells show an intermediate fluorescence intensity. *C*, Heterogeneous expression pattern of CD45RA and CD45RO within the CD3+ T-lymphocyte subset; large subpopulation of CD45RAlow/CD45RO low T-lymphocytes in neonates. *D*, Idem; higher level of CD45RO expression in adult sample. *E*, Dot plot with FITC-conjugated CD45RA McAb. *F*, Dot plot with PE-conjugated CD45RA McAb (same sample as *E*). *G*) Dot plot with FITC-conjugated CD45RO McAb. *H*, Dot plot with PE-conjugated CD45RO McAb (same sample as *G*).

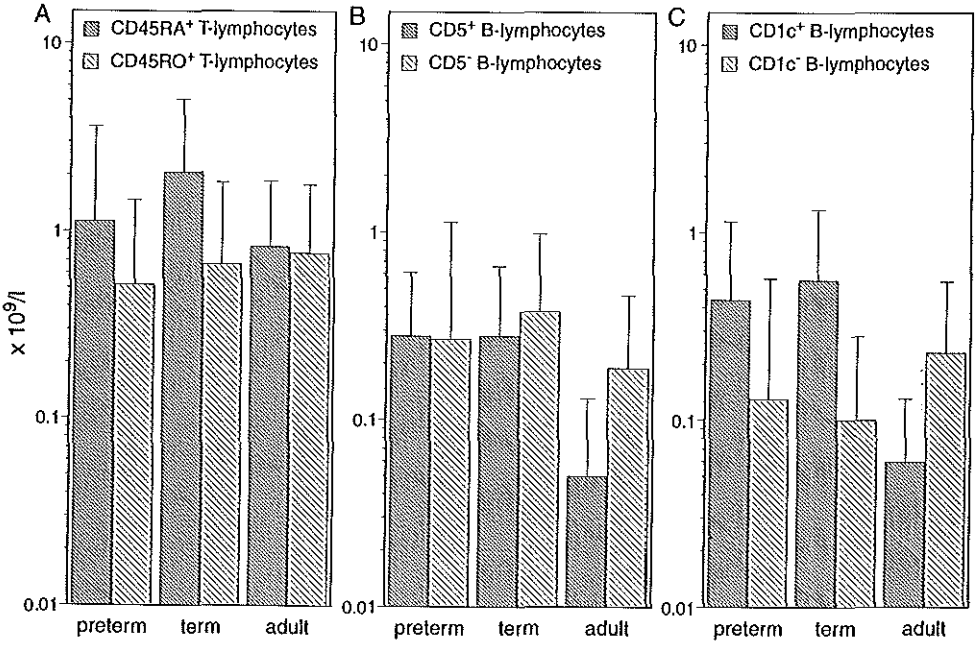

Figure 3. Comparison of absolute counts (geometrical mean and range) of several lymphocyte subpopulations in preterm neonates, term neonates, and adults. *A*, The CD45RO+ "memory" T-lymphocyte subset has a similar size in neonates and adults, but the CD45RA+ "naive" T-lymphocyte subset is much larger in the term neonates. *B*, The CD5+ B-lymphocyte subpopulation is roughly twice as large in neonates as compared to adults. *C*, The CD1c+ B-lymphocyte subpopulation is much larger in the neonates than in the adults.

The absolute counts of T- and B-lymphocytes and NK-cells in the preterm neonates were similar to those in the term neonates (Table 3).

T-lymphocyte subpopulations

The CD4/CD8 ratio was higher in term neonates as compared to adults, due to their high absolute counts of CD4+ T-lymphocytes. The CD4/CD8 ratio was highest in the preterm infants, due to their lower absolute counts of CD8+ T-lymphocytes (Tables 2 and 3).

The relative frequencies of CD45RO+ "memory" T-lymphocytes were lower in term neonates than in adults (Table 2), as has been described before.[11,12] However, the absolute counts of the CD45RO+ "memory" T-lymphocyte population were similar in both groups, whereas the absolute counts of the CD45RA+ "naive" T-lymphocyte population were larger in the term neonates as compared to the adults (median in Table 3; geometrical mean in Figure 3a). Three out of 5 preterm neonates (29, 30 and 34 weeks) did not have such a large population of CD45RA+ "naive" T-lymphocytes (Table 3; Figure 3a). However, the difference between the CD45RA+ "naive" T-lymphocyte counts between preterm neonates and term

neonates was not statistically significant (Table 3).

B-lymphocyte subpopulations

Like others,[6,7] we found that the relative frequencies of CD1c+ and CD5+ B-lymphocyte subpopulations were larger in neonates than in adults (Table 2). The geometrical mean of the CD1c+ as well as the CD5+ B-lymphocyte subpopulation absolute counts was larger in neonates than in adults (Figures 3b and 3c). The median absolute counts of both the CD5+ and the CD5- B-lymphocyte subpopulation were roughly twice as large in the preterm and term neonates as compared to the adults (Table 3). The median absolute counts of the CD1c+ B-lymphocyte subpopulation were larger in preterm and term neonates than in adults, whereas the CD1c- B-lymphocyte subpopulation was similar in size in preterm neonates, term neonates and adults (Table 3).

NK-cell subpopulations

Despite similar relative frequencies of CD8+ NK-cells in neonates and adults (Table 2), the absolute counts of both the CD8+ and the CD8- NK-cell subpopulations were larger in neonates than in adults, due to the higher absolute counts of NK-cells in neonates (Table 3).

DISCUSSION

The considerable erythroid contamination of the "lympho-gate" we found in neonatal cord blood samples leads to a high relative frequency of CD71+ cells within this "lympho-gate". If single instead of double or triple immunostaining is used, it is impossible to distinguish these CD71+ normoblasts from CD71+ lymphocytes.[7] Appropriate correction for this erythroid cell contamination of the "lympho-gate", e.g. with the CD71/GpA/CD45 triple immunostaining as we did here, prevents underestimation of the relative frequencies of lymphocyte subpopulations, and allows accurate calculation of absolute counts.[5]

Markers with heterogeneous expression patterns within particular lymphocyte subsets, like the CD45RA and CD45RO isoforms on T-lymphocytes, are difficult to analyze with flow cytometric immunophenotyping without strict guidelines for reproducible quadrant settings.[8] In addition, we found differences in the intensity of expression between the various CD45RA and CD45RO McAb conjugated to different fluorochromes, which were more pronounced in the neonatal cord blood samples. We prefered usage of PE-conjugated CD45RA and CD45RO McAb, because their greater intensity of staining enabled more accurate dot plot quadrant setting.

After addressing the technical problems described above, we were able to obtain accurate absolute counts for comparison of the actual sizes of blood lymphocyte subpopulations in preterm neonates, term neonates, and adults.

Because the neonatal immune system is truly "naive", i.e. not challenged with antigens, it is likely that the dominant lymphocyte subpopulations in neonates represent "untriggered" cells, recently emigrated from the bone marrow (B-lymphocytes) or thymus (T-lymphocytes). The higher absolute counts of T- and B-lymphocytes and NK-cells in neonates imply that *sub*populations of these cells which have lower relative frequencies in neonates than in adults can actually be similar or larger in size (i.e. have the same or higher absolute counts) in neonates. The importance of comparing the actual numbers of neonatal and adult lymphocyte subpopulations to determine which subpopulations are predominant in neonates, is illustrated by CD45RA and CD45RO expression on T-lymphocytes,[6] and CD1c and CD5 expression on B-lymphocytes.[7]

We found clear differences in the relative frequencies of CD45RO+ "memory" T-lymphocytes between neonates and adults, which is in line with previous reports.[11,12] However, the absolute numbers of the CD45RO+ "memory" T-lymphocyte population were similar in both groups, albeit with a lower intensity of staining in the neonates, whereas the absolute numbers of the CD45RA+ "naive" T-lymphocyte population were much larger in neonates as compared to adults. So, the differences in relative frequency of CD45RA+ "naive" and CD45RO+ "memory" T-lymphocytes between neonates and adults are *not* caused by a smaller CD45RO+ "memory" T-lymphocyte subpopulation, but by a larger CD45RA+ "naive" T-lymphocyte subpopulation in neonates as compared to adults. In addition, the differences we found in the intensity of staining of CD45RA and CD45RO McAb between neonates and adults, render the interpretation of comparative studies between neonates and adults difficult: the CD45RA$_{low}$/CD45RO$_{low}$ T-lymphocytes found in neonates, have no counterpart in adults. They have been presumed to be truly naive "untriggered" recent thymic emigrants.[9]

CD5+ B-lymphocytes are assumed to represent a more primitive lineage than "conventional" CD5- B-lymphocytes;[10] the role of CD1c+ B-lymphocytes is not yet clear. CD1c as well as CD5 are expressed on a higher relative frequency of B-lymphocytes in neonates than in adults,[7] which suggests they could be "untriggered" bone marrow emigrants. CD1c+ as well as CD5+ B-lymphocyte subpopulation absolute counts were larger in neonates than in adults; CD1c+ and CD5+ B-lymphocytes might indeed represent such "untriggered" B-cell subsets.

Our findings suggest that a large pool of naive "untriggered" cells is stand-by in term neonates for participation in primary immune responses. In some preterm neonates this pool was smaller than in term neonates, which might explain a higher susceptibility to infections, due to a slower or less efficient primary immune response as compared to term neonates. Antigen-triggered cells seem to be present in comparable numbers in neonates and adults, but the differences in intensity of cell surface marker expression suggest that these cells might still be functionally different in neonates and adults. This issue remains to be solved in future studies.

REFERENCES

1 Lewis DB, Wilson CB: Developmental immunology and role of host defences in neonatal susceptibility to infection; in Remington JS, Klein JO (eds): *Infectious diseases of the fetus and newborn infant.* Philadelphia (PA), Saunders, 1995, pp 20-98.
2 Oski F. The erythrocyte and its disorders; in Nathan D, Oski F (eds): *Hematology of infancy and childhood.* Philadelphia (PA), Saunders, 1993, pp 18-43.
3 Comans-Bitter WM, de Groot R, van den Beemd R, van den Beemd R, Neijens HJ, Hop WCJ, Groeneveld K, Hooijkaas H, van Dongen JJM. Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. *J Pediatr* 1997;130:388-393.
4 Loken MR, Brosnan JM, Bach BA, Ault KA. Establishing optimal lymphocyte gates for immunophenotyping by flow cytometry. *Cytometry* 1990;11:453-459.
5 De Vries E, de Bruin-Versteeg S, Comans-Bitter WM, de Groot R, Boerma GJM, Lotgering FK, van Dongen JJM. Correction for erythroid cell contamination in microassay for immunophenotyping of neonatal lymphocytes. *Arch Dis Child* 1999, in press.
6 Erkeller-Yuskel FM, Deneys V, Yuksel B, Hannet I, Hulstaert F, Hamilton C, Mac-kinnon H, Turner Stokes L, Munhyeshuli V, Vanlangendonck F, deBruyère M, Bach BA, Lydyard PM. Age-related changes in human blood lymphocyte subpopulations. *J Pediatr* 1991;120:216-222.
7 Durandy A, Thuillier L, Forveille M, Fischer A. Phenotypic and functional characteristics of human newborns' B lymphocytes. *J Immunol* 1990;144:60-65.
8 Fleisher TA. Immunophenotyping of lymphocytes by flow cytometry. *Immunol Allergy Clin* 1994;14:225-240.
9 Bofill M, Akbar AN, Salmon M, Robinson M, Burford G, Janossy G. Immature CD45RA (low) CD45RO (low) T cells in the human cord blood. I. Antecedents of CD45RA+ unprimed T cells. *J Immunol* 1994;152:5613-5623.
10 Herzenberg LA, Kantor AB, Herzenberg LA. Layered evolution in the immune system. A model for the ontogeny and develoment of multiple lymphocyte lineages. In: CD5 B cells in development and disease. Proceedings of a conference. *Ann NY Acad Sci* 1991;651:1-9.
11 Aldhous MC, Raab GM, Doherty KV, Mok JYQ, Bird AG, Froebel KS. Age-related ranges of memory, activation, and cytotoxic markers on CD4 and CD8 cells in children. *J Clin Immunol* 1994;14:289-298.
12 Kingsley G, Pitzalis C, Waugh AP, Panayi GS: Correlation of immunoregulatory function with cell phenotype in cord blood lymphocytes. *Clin Exp Immunol* 1988;73:40-45.

CHAPTER 6

LONGITUDINAL FOLLOW-UP OF BLOOD LYMPHOCYTE SUBPOPULATIONS FROM BIRTH TO ONE YEAR OF AGE

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J Pediatr 1998;133:586-588

INTRODUCTION

Recently, we described prominent age-related changes in blood lymphocyte subpopulations during the first years of life, especially during the first twelve months. These changes were only detected when absolute counts instead of relative frequencies were considered.[1] In their editorial comment in the Journal, Bonilla and Oettgen remarked that the presented age-related patterns of blood lymphocyte subset counts were measured in *populations*, not in *individuals*. Therefore they argued whether to use absolute counts or relative frequencies of lymphocyte subpopulations for the follow up of individual patients.[2]

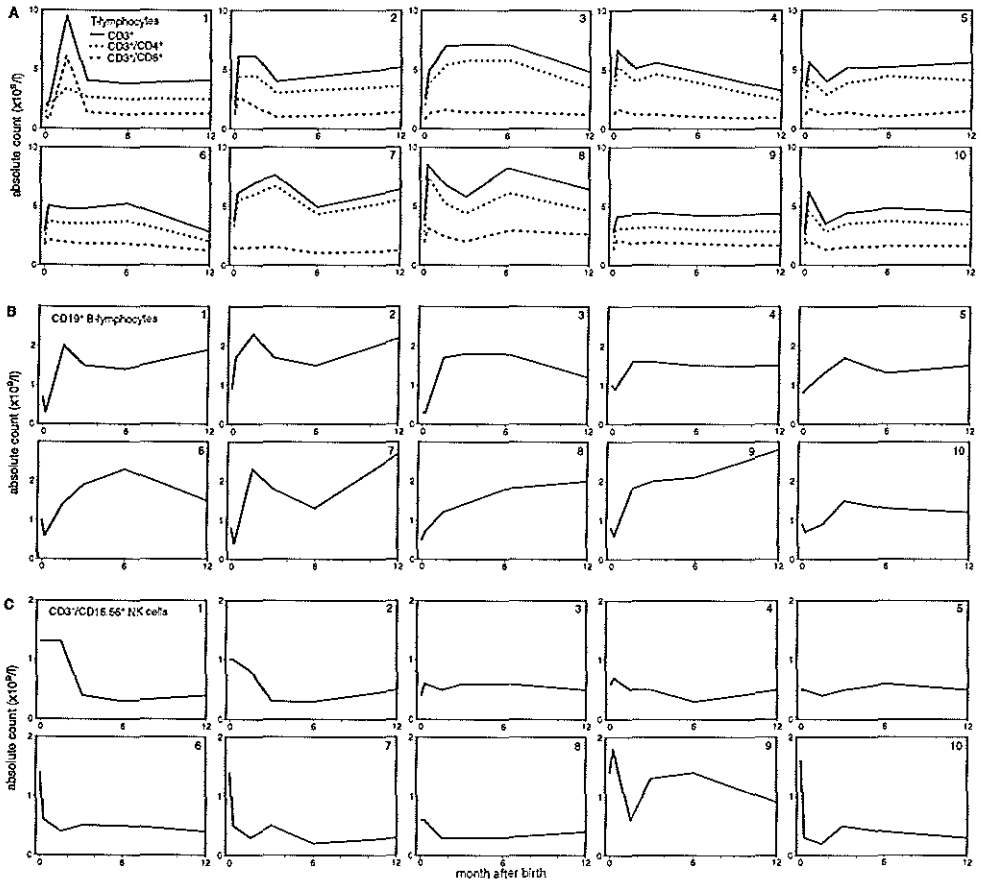

Figure 1. Absolute counts of lymphocyte subpopulations in ten children followed longitudinally from birth to one year of age. X-axis: days after birth. Y-axis: absolute count of lymphocyte subpopulation x10 ^{9}/l. Each graph represents one individual child. *A*, Absolute counts of CD3+ T-lymphocytes (-), CD3+/CD4+ (-) and CD3+/CD8+ (..) T-lymphocyte subpopulations. *B*, Absolute counts of CD19+ B-lymphocytes. *C*, Absolute counts of CD16+ and/or CD56+/CD3- NK-cells.

METHOD

We investigated blood lymphocyte subpopulation absolute counts in the blood of ten individual children followed longitudinally from birth to one year of age by immunophenotyping with a lysed whole blood microassay including a CD71/GpA/CD45 triple immunostaining to identify normoblasts and unlysed erythrocytes within the "lympho-gate".

RESULTS AND DISCUSSION

Blood lymphocyte subpopulation absolute counts of these ten children followed a similar pattern (Figure 1). The T-lymphocyte population increased in size immediately after birth. This increase mainly concerned the CD3+/CD4+ T-lymphocyte subpopulation, whereas the CD3+/CD8+ T-lymphocyte subpopulation remained relatively stable throughout the study period. These results are comparable to those obtained in our cross-sectional study.[1] B-lymphocytes also showed a rapid increase in the first six weeks of life. However, six children showed a slight decrease of their B-lymphocytes in the first week of life, before they increased in the weeks thereafter (Figure 1b). This transitory decrease was not visible in our cross-sectional study, because children aged 1 week to 2 months formed one age group.[1] These changes in B- and T-lymphocyte subsets reflect the high activity of the immune system in coping with all the antigens in the environment: B-lymphocytes start to build up an extensive antibody repertoire after birth, guided by helper T-lymphocytes.

Six children had very high NK-cell counts at birth. All ten children showed a decrease of NK-cell absolute counts in the first six weeks to three months of life (Figure 1c), as previously described in the cross-sectional study.[1] NK-cell absolute counts remained stable during the remainder of the first year of life, with the exception of one child whose NK-cell counts remained high despite a temporary decrease at six weeks of age (child nr.9 in Figure 1c). She was apparently healthy during the entire study period (Figure 1c). These findings are intriguing, but difficult to explain with our current limited knowledge of NK-cell function.

The influence of even minor infections on blood lymphocyte (sub)populations was illustrated by one child who had a very high CD3+/CD8+ T-lymphocyte count at 6 weeks of age (child nr.1 in Figure 1a): she suffered from her first - probably viral - upper respiratory tract infection at that time.

Our observations confirm the need to perform diagnostic immunophenotyping of lymphocyte subpopulations in a clinically stable phase with the use of appropriate age-matched reference values.

REFERENCES

1 Comans-Bitter WM, de Groot R, van den Beemd R, et al. Immunophenotyping of blood

lymphocytes in childhood. Reference values for lymphocyte subpopulations. *J Pediatr* 1997;130:388-393.
2 Bonilla FA, Oettgen HC. Normal ranges for lymphocyte subsets in children. *J Pediatr* 1997;130:347-349.

CHAPTER 7

LONGITUDINAL SURVEY OF LYMPHOCYTE SUBPOPULATIONS IN THE FIRST YEAR OF LIFE

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Submitted

ABSTRACT

Age-matched reference values for lymphocyte subpopulations are generally obtained via cross-sectional studies, whereas patients are followed longitudinally. We performed a detailed longitudinal analysis of the changes in lymphocyte subpopulations in a group of eleven healthy infants followed from birth up to one year of age, with special attention for early developmental markers, markers of maturation, and markers of activation.

We found that T- and B-lymphocytes increased at 1 and 6 weeks of age, respectively, whereas NK-cells showed a sharp decline directly after birth.

CD45RA+ - mainly CD4+ - "naive" T-lymphocytes were high at birth, and increased further during the first year of life; they form a large expanding pool of cells, ready for participation in primary immune responses. The absolute counts of CD45RO+ "memory" T-lymphocytes were similar in infants and adults, albeit with a lower level of expression of CD45RO on infant T-lymphocytes. Almost all infant T-lymphocytes expressed CD38 throughout the first year of life. CD38 is known to be present on thymocytes and activated T-lymphocytes, but we did not find any evidence for a premature release of "thymocyte-like" T-cells into the circulation in infants, nor did we find an increased expression of other activation markers. The abundant expression of CD38 on infant's T-lymphocytes might be related to a greater metabolic need of the large population of naive "untriggered" cells which are continually involved in primary immune responses during the first year of life.

The high B-lymphocyte counts in infants mainly concerned CD38+ B-lymphocytes throughout the first year of life. Also, the relative frequencies of CD1c+ and CD5+ B-lymphocytes were higher throughout the first year of life than in adults. So, CD1c, CD5, and CD38 could be markers of "untriggered" B-lymphocytes.

NK-cells were high at birth and declined soon afterwards; they seem to be more important during pregnancy than thereafter. The high NK-cell count at birth was for a large part accounted for by CD8+ NK-cells. Also, at birth some infants showed large subpopulations of CD7-, CD38-, and CD45RA- NK-cells.

In conclusion, our longitudinal survey of T- and B-lymphocytes, NK-cells, and their subpopulations during the first year of life helps to complete the picture of lymphocyte development in infants. This information contributes to the correct interpretation of data from infants with possible immune disorders.

INTRODUCTION

Immunophenotyping of blood lymphocyte subpopulations is an important tool in the diagnosis and follow-up of children with immune disorders. Correct interpretation of the obtained results requires knowledge of the normal

development of the immune system during the first years of life.

For this purpose, several sets of age-matched reference values of relative frequencies and absolute counts of lymphocyte subpopulations in childhood have been reported.[1-5] Because of the higher blood lymphocyte counts in neonates and infants as compared to adults,[1,6] differences in lymphocyte subpopulations are better reflected by comparison of absolute counts than relative frequencies. In that way, trends are observed which are missed when only relative frequencies are used.[1] These absolute lymphocyte counts are more accurately determined by the whole lysed blood technique than by analysis after density gradient separation.[7,8]

To date, age-matched reference values for lymphocyte subpopulations were all obtained in cross-sectional studies. Longitudinal studies in individual children are more informative about the pattern of lymphocyte subpopulation development in time. Children with immune disorders are also followed longitudinally. Therefore, it is useful to compare patient data with data from studies on longitudinal development of lymphocyte subpopulations in healthy children. Such longitudinal follow-up studies can also assess the influence of factors like infection and vaccination on the size of lymphocyte subpopulations. This information is important for correct timing of lymphocyte subpopulation studies for diagnostic purposes as well as for disease monitoring in patients.

We therefore performed a detailed longitudinal analysis of lymphocyte subpopulations by immunophenotyping with a lysed whole blood microassay in a group of eleven healthy infants followed from birth to one year of age. We studied helper- and cytotoxic-T-lymphocytes, B-lymphocytes, and NK-cells, and their subpopulations. Because the presence of immature as well as of activated cells has been described in neonatal samples in the past,[9] special attention was paid to the expression of early developmental markers, markers of maturation, and markers of activation.

MATERIALS AND METHODS

Subjects and cell samples

Immediately after clamping of the cord, neonatal cord blood was collected by venipuncture from eleven healthy infants born after an uncomplicated pregnancy and delivery at gestational ages of 35 weeks (infant no. 10), 37 weeks (infant no. 6), 38 weeks (infant no. 4), 40 weeks (infants no. 2, 3, 9, and 11), and 41 weeks (infants no. 1, 5, 7, and 8). At the age of 7 days, 6 weeks, 3 months, 6 months, and 1 year additional blood samples were collected by venipuncture from the same infants (infant nr.11 was lost from follow-up at 6 months of age). The blood was kept at room temperature until immunostaining was performed within 12 hours after sampling. Every time a blood sample was taken, a physical examination of the infant was performed by the pediatrician (EV), and questions about infections, vaccinations, feedings, and development were answered by the

mother. Adult peripheral blood was drawn by venipuncture from nine healthy adult volunteers (age 20 to 40 years). Ethylene-diamino-tetra-acetate was used as anti-coagulant. Informed consent was obtained according to the guidelines of the Medical Ethics Committee of the Erasmus University Rotterdam/University Hospital Rotterdam.

Flow cytometric analysis

The monoclonal antibodies (McAb) used in thirty triple labelings (listed in Table 1) were conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), or the duochrome PE-Cyanin5 (PE-Cy5). The same set of thirty triple immunostainings was used in all samples, to enable accurate comparison of the relative and absolute sizes of the different T-lymphocyte (CD3+), B-lymphocyte (CD19+), and NK-cell (CD16+ and/or CD56+ /CD3-) subpopulations between the infants at the various time-points in their first year of life and the adults.

A lysed whole blood microassay was used for all stainings as described previously.[10] Briefly, 20 µl of blood was incubated with three optimally titrated McAb for 10 min at room temperature. Erythrocytes were lysed with Lysing Solution (Becton Dickinson, San Jose, CA). The CD71/GpA/CD45 triple immunostaining was included to identify normoblasts and unlysed erythrocytes within the "lympho-gate".[10] The samples were analyzed with a FACScan flow cytometer, using FACScan Research software (Becton Dickinson, San Jose, CA).

Lymphocyte count and differentiation

The total nucleated cell count was determined on an H1 Technicon hemocytometer (Bayer, Tarrytown, NY). The relative frequency of normoblasts was determined by manual differentiation of 400 nucleated cells in all samples. The total lymphocyte count was determined according to the following formula:

total nucleated cell count x (100 - % of normoblasts) x (% of lymphocytes) x 10^{-4}.

Lymphocyte subpopulations were calculated according to the following formula:

$$\frac{\%\text{ of population within the "lympho-gate"}}{100 - (\%\text{ of normoblasts plus unlysed erythrocytes within the "lympho-gate"})} \times 10^2$$

Statistics

After applying logarithmic transformation to obtain approximately normally distributed variables, repeated measurements ANOVA was used to compare the various time-points. Additional specific comparisons were made for T-lymphocytes, NK-cells and their subpopulations in cord blood versus the mean of these parameters at the other time-points. Also, for B-lymhocytes and their subpopulations the mean of cord blood and 1 week was compared to the mean of the time-points thereafter. To evaluate the effects of infection, vaccination, medication, and breast feeding at the various time-points the t-test was used for each factor. In view of the number of comparisons made, the significance level was set at $p<0.01$.

TABLE 1. Thirty triple immunostainings used in the longitudinal study.

Marker	FITC-conjugated McAb (company[a])	Marker	PE-conjugated McAb (company[a])	Marker	PE-Cy5 conjugated McAb (company[a])
IgG1	IgG1 (BD)	IgG2	IgG2 (BD)	IgG1	679.1Mc7 (IT)
CD15	CLB-gran2 (CLB)	CD14	My-4 (CT)	CD45	HI30 (PH)
CD71	66IG10 (MS)	GpA	D2.10 (PH)	CD45	HI30 (PH)
CD37	IOB1 (IT)	CD20	Leu-16 (BD)	CD19	HIB19 (PH)
CD5	Leu-1 (BD)	CD19	Leu-12 (BD)	CD3	Hit3a (PH)
CD38	IOB6 (IT)	CD45RA	2H4 (CT)	CD19	HIB19 (PH)
CD3	Leu-4 (BD)	CD1a	T6 (CT)	CD19	HIB19 (PH)
CD1c	7C6 (KM)	CD19	Leu-12 (BD)	CD3	Hit3a (PH)
CD25	2A3 (BD)	CD19	Leu-12 (BD)	CD3	Hit3a (PH)
CD71	66IG10 (MS)	CD19	Leu-12 (BD)	CD3	Hit3a (PH)
CD4	Leu-3a (BD)	CD8	Leu-2a (BD)	CD3	Hit3a (PH)
TCRαβ	WT31 (BD)	CD4	Leu-3a (BD)	CD3	Hit3a (PH)
TCRαβ	WT31 (BD)	CD8	Leu-2a (BD)	CD3	Hit3a (PH)
TCRγδ	TCRδ1 (TD)	CD4	Leu-3a (BD)	CD3	Hit3a (PH)
TCRγδ	TCRδ1 (TD)	CD8	Leu-2a (BD)	CD3	Hit3a (PH)
CD4	Leu-3a (BD)	CD45RA	2H4 (CT)	CD3	Hit3a (PH)
CD8	Leu-2a (BD)	CD45RA	2H4 (CT)	CD3	Hit3a (PH)
CD4	Leu-3a (BD)	CD45RO	UCHL-1 (DK)	CD3	Hit3a (PH)
CD8	Leu-2a (BD)	CD45RO	UCHL-1 (DK)	CD3	Hit3a (PH)
CD45RO	UCHL-1 (DK)	CD45RA	2H4 (CT)	CD3	Hit3a (PH)
CD38	IOB6 (IT)	CD45RO	UCHL-1 (DK)	CD3	Hit3a (PH)
CD38	IOB6 (IT)	CD45RA	2H4 (CT)	CD3	Hit3a (PH)
CD57	Leu-7 (BD)	CD16/CD56	Leu-11c/Leu-19 (BD)	CD3	Hit3a (PH)
CD7	CLB3A1/1 (CLB)	CD16/CD56	Leu-11c/Leu-19 (BD)	CD3	Hit3a (PH)
CD8	Leu-2a (BD)	CD16/CD56	Leu-11c/Leu-19 (BD)	CD3	Hit3a (PH)
CD38	IOB6 (IT)	CD16/CD56	Leu-11c/Leu-19 (BD)	CD3	Hit3a (PH)
CD45RO	UCHL-1 (DK)	CD16/CD56	Leu-11c/Leu-19 (BD)	CD3	Hit3a (PH)
CD45RA	Leu-18 (BD)	CD16/CD56	Leu-11c/Leu-19 (BD)	CD3	Hit3a (PH)
CD25	2A3 (BD)	CD16/CD56	Leu-11c/Leu-19 (BD)	CD3	Hit3a (PH)
HLA-DR	L243 (BD)	CD16/CD56	Leu-11c/Leu-19 (BD)	CD3	Hit3a (PH)

[a] Companies: BD = Becton Dickinson, San Jose, CA; CLB = Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands; MS = Monosan, Sanbio, Uden, The Netherlands; IT = Immunotech, Marseille, France; KM = Dr. W. Knapp and dr. O. Madjic, Vienna, Austria; TD = T-cell Diagnostics, Cambridge, MA; DK = Dakopatts, Glostrup, Denmark; CT = Coulter Clone, Hialeah, FL; PH = Pharmingen, San Diego, CA.

RESULTS

Subject follow-up

All infants were born after a normal pregnancy and delivery. All had birth weights appropriate for gestational age. None of the infants used medication at any time-point when a blood sample was taken. Several infants suffered from minor infections of the upper airways or gastrointestinal tract around the time a blood sample was taken. One infant developed atopic eczema and food allergies (infant nr.5). No other medical problems were noted. The infants were vaccinated according to the regular Dutch schedule, which consisted of diphteria toxoid, tetanus toxoid, pertussis, and inactivated polio virus type I, II, and III (DTP-IPV) at 3, 4, 5, and 11 months of age at the time of the study.

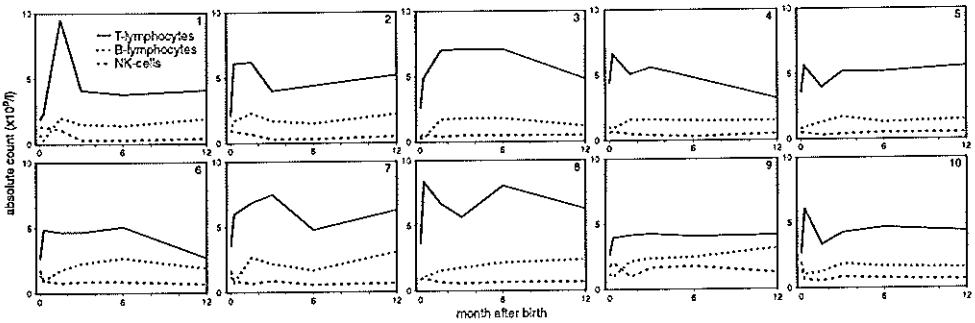

Figure 1. T-lymphocytes, B-lymphocytes, and NK-cells from birth to 1 year of age. Each graph represents one individual infant (infants no. 1-10).

Lymphocyte counts

Total lymphocyte counts and T-lymphocyte counts increased at 1 week of age. B-lymphocyte counts increased at 6 weeks of age. NK-cells followed a different pattern: they were highest at birth and decreased at 1 week of age (Figure 1, Tables 2-4).

TCR-expression on T-lymphocytes

Most T-lymphocytes expressed the αβ T-cell receptor (TCR) (Table 5). TCRγδ+ T-lymphocyte counts increased slightly during the first year of life (Table 2).

Expression of CD4 and CD8 on T-lymphocytes

CD4+ helper T-lymphocyte counts followed the pattern of total T-lymphocyte counts with an increase at 1 week of age, as did CD8+ cytotoxic T-lymphocyte counts (Table 2). Although both CD4+/CD3+ and CD8+/CD3+ counts were higher in infants than in adults, the CD4/CD8 ratio was higher in infants than in adults, due to the very high CD4+/CD3+ counts in infants (Tables 2 and 5).

Hardly any "thymocyte-like" T-cells

In the thymus, CD4+/CD8+ "double-positive" T-cells develop into "single-positive" CD4+ or CD8+ T-lymphocytes,[11] and CD1a is a marker present on cortical thymocytes.[12] Hardly any CD4+/CD8+ "double-positive" or CD1a+ T-cells were present in the blood of either infants or adults (Figure 2a-d, Tables 2 and 5).

CD45 isoform expression on T-lymphocytes

The relative expression of the CD45RA and CD45RO isoforms was profoundly different between infants and adults: the median relative frequencies of CD45RA+ "naive" T-lymphocytes were higher, and the median relative frequencies of CD45RO+ "memory" T-lymphocytes were lower in infants than in adults (Table 5), as was described before.[3-5,12] However, the median *absolute counts* of CD45RO+ "memory" T-lymphocytes were *similar* in infants and adults, with a slight increase at 1 week of age (Table 2). The changes in the absolute counts of T-lymphocytes during the first year of life were mainly accounted for by changes in the size of the CD45RA+ "naive" T-lymphocyte population, *not* by changes in the size of the

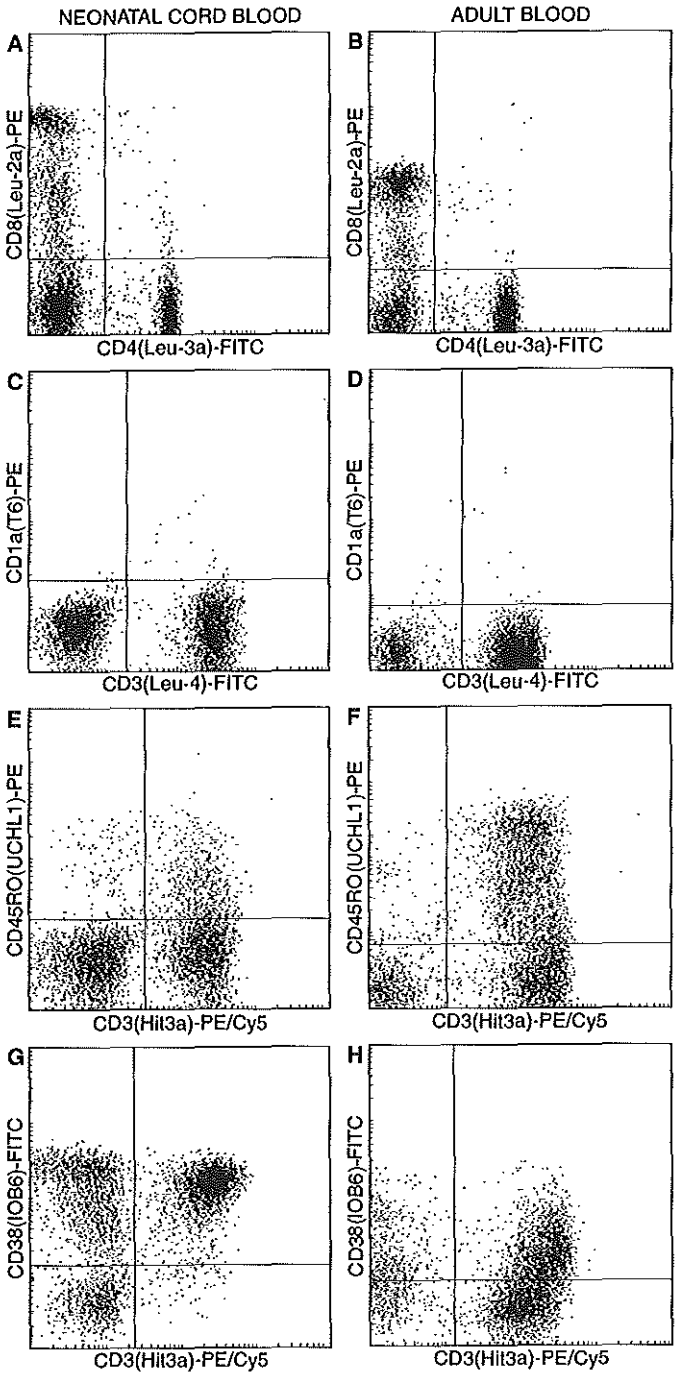

Figure 2. Expression of early developmental markers and markers of maturation by T-lymphocytes. Hardly any CD4+/CD8+ "double-positive" T-cells were present in neonates (*A*) and adults (*B*). CD1a was hardly expressed on T-cells at birth (*C*), or in adults (*D*). The intensity of staining of CD45RO+ "memory" T-lymphocytes was lower in neonates (*E*) than in adults (*F*). CD38 was expressed by most T-lymphocytes in neonates (*G*), and by about half of adult T-lymphocytes (*H*).

CD45RO+ "memory" T-lymphocyte population in the course of time (Figures 3 and 4). However, the intensity of CD45RO staining was higher in adults than in infants, i.e. adult T-lymphocytes probably carried more CD45RO molecules on their cell surface (Figure 2e/f).

The increase in CD45RA+ "naive" T-lymphocytes during the first year of life and the slight increase of CD45RO+ "memory" T-lymphocytes at 1 week of age was due to an increase in CD4+ helper T-lymphocytes as well as CD8+ cytotoxic T-lymphocytes (Tables 2 and 5).

Expression of CD38 on T-lymphocytes

We found CD38 on ≥90% (median) of T-lymphocytes in infants, and on about half of adult T-lymphocytes (Figure 2g/h; Table 5).

The distribution of CD38 on CD45RA+ "naive" and CD45RO+ "memory" T-lymphocytes was markedly different between infants and adults (Table 5). In adults, the CD38+/CD45RA+/CD3+ and the CD38+/CD45RO+/CD3+ subpopulations were much smaller, and the CD38-/CD45RA+/CD3+ and the CD38-/CD45RO+/CD3+ subpopulations were much larger than in infants. The changes in size of the CD45RA+ "naive" T-lymphocyte subpopulation in the first year of life were mainly due to changes in size of the CD38+/CD45RA+/CD3+ subpopulation. The median absolute counts of CD38-/CD45RO+/CD3+ T-lymphocytes increased only slightly during the first year of life. The slight peak of CD45RO+ "memory" T-lymphocytes at 1 week of age was mainly caused by an increase in CD38+/CD45RO+/CD3+ T-lymphocytes (Table 2).

The expression of CD38 on CD4+ helper T-lymphocytes and on CD8+ cytotoxic T-lymphocytes was also different between infants and adults (Table 5). In adults, most CD8+ T-lymphocytes were CD38-, whereas about half of CD4+ T-lymphocytes was CD38+. The changes in CD4+ T-lymphocytes in the first year of life were mainly caused by changes in the size of the CD38+/CD4+/CD3+ subpopulation, not by changes in the size of the CD38-/CD4+/CD3+ subpopulations (Table 2).

Expression of CD25 and HLA-DR on T-lymphocytes

The absolute counts of T-lymphocytes bearing CD25 (the IL-2 receptor α chain) on their surface peaked at 1 week of age. The absolute counts of HLA-DR+ T-lymphocytes did not. They were lower than in adults throughout the first year of life (Table 2).

Absence of CD57+ T-lymphocytes

The human natural killer cell antigen, CD57, is present on a subpopulation of T-lymphocytes. It has been suggested that these cells represent previously activated T-lymphocytes, which have returned to a state of rest.[14] CD57+ T-lymphocytes were virtually absent at birth, and very low up to 6 months of age, except for two infants who had higher values at some, but not all, time-points (Tables 2 and 5).

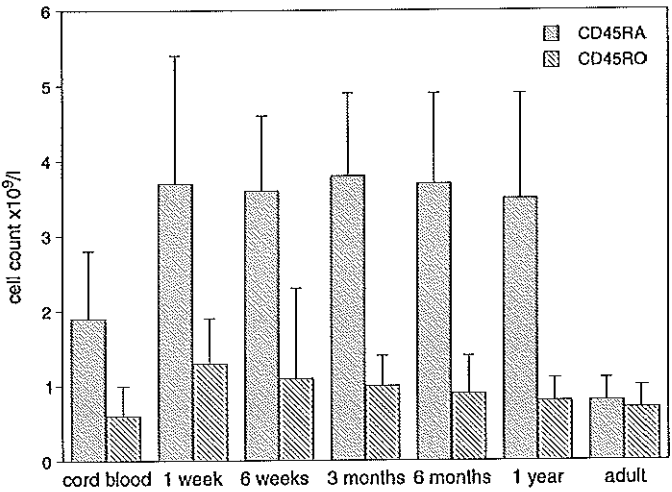

Figure 3. Absolute counts (geometrical mean and standard deviation) of CD45RA+ and CD45RO+ T-lymphocyte subpopulations x 10^9/l in 11 infants during the first year of life and in 9 adults. Left bars represent CD45RA+ T-lymphocytes and right bars represent CD45RO+ T-lymphocytes.

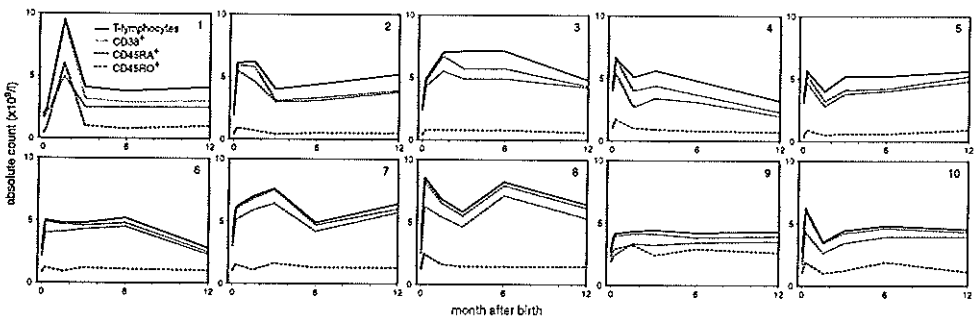

Figure 4. CD45RA+ and CD38+ T-lymphocytes followed the pattern of total T-lymphocytes in the infants, but CD45RO+ T-lymphocytes did not. Each graph represents one individual infant (infants nrs. 1-10).

Hardly any precursor-B-cells

There was no indication that B-lymphocytes leave the bone marrow at an earlier developmental stage in infants than in adults: hardly any CD37-/CD20-/CD19+ precursor-B-cells[15] were present in the samples of either infants or adults (Tables 3 and 6).

Expression of CD1c, CD5 and CD38 on B-lymphocytes

The relative frequencies of CD1c+ and CD5+ B-lymphocytes were higher in infants than in adults. CD1c+ and CD5+ B-lymphocyte absolute counts followed the pattern of total B-lymphocytes during the first year of life with high values from

6 weeks of age onwards. The same was observed for CD1c- and CD5- B-lymphocyte absolute counts, although this was not statistically significant in the latter (Figure 5, Table 3). CD38 was present on a median of >90% of B-lymphocytes throughout the first year of life, and on a median of 82% of B-lymphocytes in adults (Table 6). CD38- B-lymphocyte absolute counts were low in both infants and adults (generally <0.1 x 10^9/l) (Table 3).

Expression of CD25 on B-lymphocytes

CD25 is a marker of activation in B-lymphocytes.[16] The relative frequencies and absolute counts of CD25+ B-lymphocytes were lower during the first year of life than in adults (Tables 3 and 6).

Expression of CD71 on B-lymphocytes

The transferrin receptor CD71 is present on a subpopulation of B-lymphocytes.[17] It is not clear what role CD71 has on the surface of B-lymphocytes. It might be present as a marker of activation as well. The absolute counts of CD71+ B-lymphocytes were the same in infants throughout the first year of life and in adults (Table 3). It is important to note that most CD71+ cells (66-95%) within the "lympho-gate" of the neonatal cord blood samples were normoblasts, as we described before.[10]

CD7-, CD38-, and CD45RA- NK-cells

At birth, some infants showed large subpopulations of NK-cells that were not expressing CD7, CD38 and CD45RA. At 1 week of age 5 out of 11, 4 out of 11, and 3 out of 11 infants still did not express CD7, CD38, and CD45RA on >20% of their NK-cells, respectively. However, the CD7-, CD38-, and CD45RA- NK-cells do not account for the high absolute NK-cell counts during the first week of life (Table 7). Only CD8+ NK-cells were significantly higher at birth than thereafter (see below).

Absence of CD57+ NK-cells

CD57 is a well-known marker of a subpopulation of NK-cells, which role is not yet known. It is rapidly lost after NK-cell activation.[18] Interestingly, CD57 was hardly expressed by NK-cells until 3 months of age (Figure 6a). From 3 months until 1 year of age a growing number of infants showed a CD57+ NK-cell subpopulation of up to around 20% (median) of NK-cells. Adults always showed a CD57+ NK-cell subpopulation (Figure 6b, Tables 4 and 7).

Absence of CD45RO+ NK-cells

The CD45RA- NK-cells in neonatal cord blood must express another isoform of the CD45 molecule, such as CD45RB or CD45RC, as almost none of these NK-cells were expressing the CD45RO isoform either (Figure 6c, Tables 4 and 7). Possibly, the small but clearly present population of CD45RO+ NK-cells in adults (Figure 6d) represents NK-cells that have encountered a stimulus, like CD45RO+ "memory" T-lymphocytes.

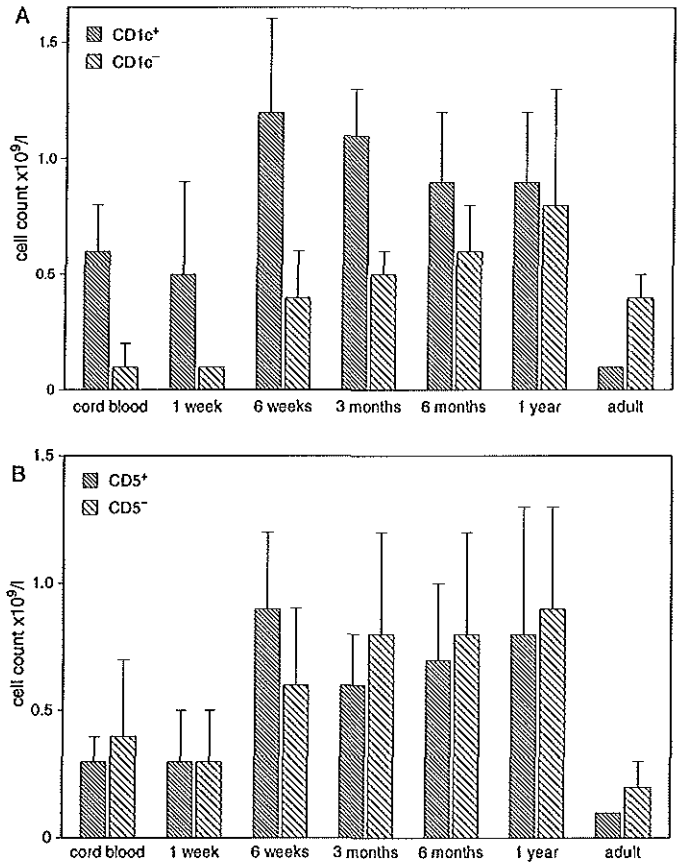

Figure 5. Absolute counts (geometrical mean and standard deviation) of various B-lymphocyte subpopulations x 10^9/l in 11 infants during the first year of life and in 9 adults. *A*, Left bars represent CD1c+ B-lymphocytes and right bars represent CD1c- B-lymphocytes. *B*, Left bars represent CD5+ B-lymphocytes and right bars represent CD5- B-lymphocytes.

Expression of CD25 and HLA-DR on NK-cells

CD25 is upregulated in NK-cells upon activation, as is HLA-DR.[19] CD25 as well as HLA-DR were hardly expressed by NK-cells in infants as well as adults (Tables 4 and 7).

Expression of CD8 on NK-cells

The relative frequency of CD8+ NK-cells did not change significantly during the first year of life (Table 7), but the absolute counts of CD8+ NK-cells at birth and 1 week of age were high (Table 4). These high CD8+ NK-cell counts can influence the determination of the CD4+/CD8+ ratio of T-lymphocytes if single immunostaining is used instead of multiple immunostaining with CD4, CD8 and CD3 McAb.

Influence of infections and vaccinations

Infant no. 1 suffered from her first, probably viral, upper respiratory tract infection at 6 weeks of age; she was not very ill, and recovered without treatment within a week. None of the other infants suffered from an infection in the first 6

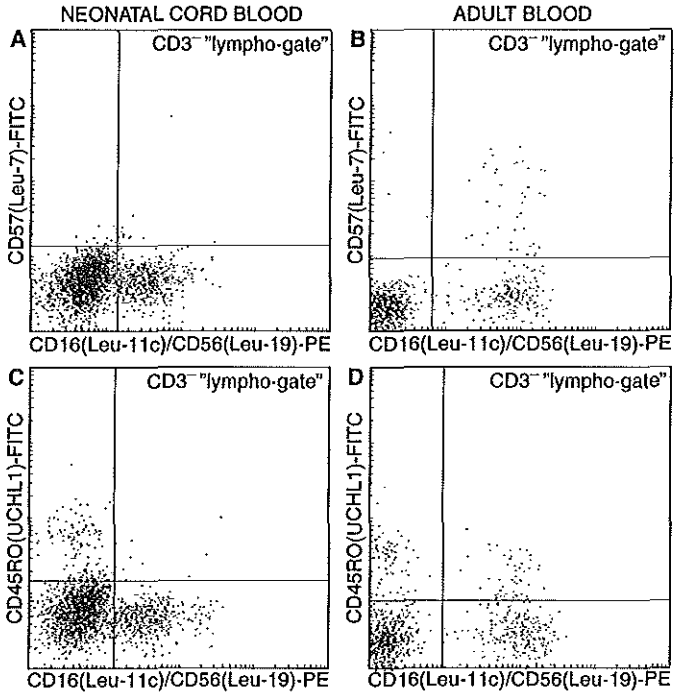

Figure 6. NK-cell subpopulations in neonates and adults. Absence of CD57+ NK-cells shortly after birth (*A*), and a clear subpopulation of CD57+ NK-cells in adults (*B*). Hardly any CD45RO+ NK-cells were present in neonates (*C*), a small subpopulation of CD45RO+ NK-cells was present in adults (*D*).

weeks of life. Infant no. 1 had a very high T-lymphocyte count at week 6 (Figure 1), due to an extremely high absolute count of CD8+ T-lymphocytes (5.8 x 10^9/l); her absolute count of CD4+ T-lymphocytes was not different from the other infants (3.3 x 10^9/l). Her CD45RO+ "memory" T-lymphocytes were high at that time (6.1 x 10^9/l); there was no significant relation between the absolute count of CD45RO+ "memory" T-lymphocytes and infection at any other time-point in our small group of infants, however. Interestingly, CD25+ T-lymphocytes in infant no. 1 were not different from the other infants (9% of T-lymphocytes; 0.8 x 10^9/l), whereas the HLA-DR+ T-lymphocyte count was very high (70% of T-lymphocytes; 6.6 x 10^9/l), as was the HLA-DR+ NK-cell count (40% of NK-cells, 0.5 x 10^9/l).

At no other time point a significant relation was found between recent infection or vaccination and the distribution of lymphocyte subpopulations in this small group of eleven infants.

DISCUSSION

After differentiation in the bone marrow and thymus, lymphocytes undergo further functional maturation in the periphery. This process is directed by numerous encounters with environmental antigens which take place from birth onwards. The immune system thus gradually builds up a pool of experienced

TABLE 2. Absolute counts of lymphocytes, CD3+ T-lymphocytes, and CD3+ T-lymphocyte subpopulations in eleven infants during follow-up from birth to one year of age and in nine adults.

	neonatal cord blood	1 week of age	6 weeks of age	3 months of age	6 months of age	1 year of age	ncb < as compared to 1wk-1yr[b]	adults
Total lymphocytes	4.7 (3.5-6.2)[a]	6.9 (4.0-9.6)	7.2 (4.1-12.6)	7.1 (5.8-9.6)	6.8 (5.6-9.9)	7.1 (4.1-8.9)	p < 0.001	2.1 (1.1-2.4)
$CD3^+$ T-lymphocytes	2.6 (1.9-4.4)	5.6 (2.4-8.0)	5.1 (3.0-9.5)	4.5 (3.9-7.1)	4.5 (3.7-7.7)	4.4 (2.3-5.9)	p < 0.001	1.5 (0.7-1.8)
T-cell subpopulations:								
$TcR\alpha\beta^+/CD3^+$	2.5 (1.8-4.3)	5.5 (2.2-7.7)	4.9 (2.9-8.5)	4.2 (3.5-7.0)	4.2 (3.3-7.5)	4.0 (2.0-5.8)	p < 0.001	1.4 (0.6-1.7)
$TcR\gamma\delta^+/CD3^+$	0.1 (0.0-0.1)	0.1 (0.0-0.2)	0.1 (0.1-0.8)	0.2 (0.1-0.3)	0.2 (0.1-0.3)	0.2 (0.1-0.3)	p < 0.001	0.1 (0.0-0.2)
$CD4^+/CD3^+$	2.0 (1.4-3.5)	4.1 (2.0-6.1)	3.3 (2.2-5.4)	3.4 (2.6-6.1)	3.5 (2.4-5.5)	3.2 (1.5-5.0)	p < 0.001	1.0 (0.4-1.1)
$CD8^+/CD3^+$	0.6 (0.5-1.0)	1.3 (0.4-2.4)	1.2 (0.7-5.8)	1.0 (0.8-1.4)	1.0 (0.5-2.2)	1.0 (0.6-1.5)	p < 0.001	0.5 (0.3-0.8)
$CD4^+/CD8^+/CD3^+$	0.1 (0.0-0.1)	0.1 (0.0-0.1)	0.1 (0.0-0.4)	0.1 (0.0-0.2)	0.0 (0.0-0.2)	0.0 (0.0-0.2)	n.s.	0.0 (0.0-0.1)
$CD1a^+/CD3^+$	0.1 (0.0-0.3)	0.0 (0.0-0.3)	0.0 (0.0-0.5)	0.0 (0.0-0.2)	0.0 (0.0-0.0)	0.0 (0.0-0.1)	n.s.	0.0 (0.0-0.1)
$CD45RA^+/CD3^+$	1.7 (1.0-3.9)	4.3 (1.9-5.5)	3.6 (2.1-5.2)	3.8 (2.7-5.8)	3.7 (2.8-6.7)	3.8 (1.8-5.1)	p < 0.001	0.9 (0.3-1.1)
$CD45RO^+/CD3^+$	0.6 (0.3-1.8)	1.2 (0.7-2.4)	0.9 (0.4-6.1)	1.1 (0.6-1.9)	0.8 (0.6-2.6)	0.8 (0.5-2.1)	p < 0.001	0.8 (0.5-1.0)
$CD38^+/CD3^+$	2.5 (1.8-4.3)	5.4 (2.2-7.7)	4.8 (2.9-9.3)	4.1 (3.6-7.0)	4.2 (3.3-7.4)	4.0 (2.0-5.6)	p < 0.001	0.7 (0.3-0.9)
$CD4^+/CD45RA^+/CD3^+$	1.2 (0.6-3.1)	2.4 (0.1-4.0)	2.3 (1.3-4.2)	2.8 (1.9-4.8)	2.9 (1.9-4.9)	2.7 (1.1-4.3)	p < 0.001	0.4 (0.1-0.8)
$CD8^+/CD45RA^+/CD3^+$	0.6 (0.4-0.8)	1.1 (0.4-1.7)	1.0 (0.6-2.5)	0.9 (0.7-1.2)	0.9 (0.5-1.9)	1.0 (0.6-1.3)	p < 0.001	0.3 (0.1-0.6)
$CD4^+/CD45RO^+/CD3^+$	0.4 (0.2-1.2)	1.0 (0.1-1.9)	0.7 (0.3-2.2)	0.7 (0.4-1.6)	0.6 (0.5-2.3)	0.6 (0.4-1.5)	p < 0.001	0.6 (0.3-0.7)
$CD8^+/CD45RO^+/CD3^+$	0.1 (0.1-0.5)	0.2 (0.1-0.7)	0.1 (0.1-4.8)	0.2 (0.1-0.5)	0.1 (0.1-0.7)	0.1 (0.1-0.5)	p = 0.002	0.2 (0.2-0.4)
$CD38^-/CD45RA^+/CD3^+$	0.1 (0.0-0.1)	0.1 (0.0-0.2)	0.1 (0.0-0.2)	0.1 (0.1-0.2)	0.1 (0.1-0.2)	0.2 (0.1-0.3)	p < 0.001	0.4 (0.2-0.7)
$CD38^+/CD45RA^+/CD3^+$	1.8 (1.1-3.7)	4.2 (1.9-5.4)	3.5 (2.0-5.1)	3.6 (2.4-5.8)	3.5 (2.7-6.4)	3.6 (1.6-4.9)	p < 0.001	0.5 (0.2-0.7)
$CD38^-/CD45RO^+/CD3^+$	0.1 (0.0-0.2)	0.1 (0.1-0.2)	0.2 (0.1-0.3)	0.1 (0.1-0.3)	0.2 (0.1-0.4)	0.2 (0.1-0.3)	p < 0.001	0.6 (0.4-0.8)
$CD38^+/CD45RO^+/CD3^+$	0.5 (0.3-1.6)	1.1 (0.6-2.2)	0.8 (0.4-6.0)	0.8 (0.5-1.5)	0.7 (0.5-2.0)	0.6 (0.3-2.1)	p < 0.001	0.3 (0.1-0.4)
$CD38^-/CD4^+/CD3^+$	0.1 (0.0-0.1)	0.1 (0.1-0.2)	0.2 (0.1-0.2)	0.2 (0.1-0.2)	0.2 (0.1-0.2)	0.2 (0.1-0.2)	p < 0.001	0.1 (0.0-0.5)
$CD38^+/CD4^+/CD3^+$	1.9 (1.3-3.5)	4.0 (1.9-6.0)	3.1 (2.2-5.3)	3.2 (2.4-6.0)	3.4 (2.3-5.4)	3.0 (1.3-4.8)	p = 0.002	0.5 (0.2-0.8)
$CD38^-/CD8^+/CD3^+$	0.1 (0.0-0.1)	0.1 (0.0-0.2)	0.1 (0.0-1.2)	0.1 (0.0-0.1)	0.1 (0.0-0.2)	0.2 (0.0-0.3)	p = 0.001	0.1 (0.0-0.8)
$CD38^+/CD8^+/CD3^+$	0.6 (0.5-0.9)	1.3 (0.4-2.2)	1.0 (0.5-5.4)	1.0 (0.7-1.3)	0.9 (0.5-2.1)	0.8 (0.5-1.3)	n.s.	0.1 (0.0-0.1)
$CD25^+/CD3^+$	0.2 (0.2-0.4)	0.7 (0.5-0.8)	0.5 (0.3-0.8)	0.4 (0.3-0.7)	0.4 (0.3-0.5)	0.3 (0.2-0.5)	p < 0.001[c]	0.4 (0.2-0.5)
$HLA\text{-}DR^+/CD3^+$	0.2 (0.0-0.9)	0.2 (0.1-1.6)	0.2 (0.1-6.6)	0.1 (0.1-1.3)	0.1 (0.1-0.2)	0.1 (0.1-0.3)	n.s.	0.4 (0.0-0.8)
$CD57^+/CD3^+$	0.0 (0.0-0.0)	0.0 (0.0-0.1)	0.0 (0.0-0.8)	0.0 (0.0-0.5)	0.0 (0.0-0.3)	0.1 (0.0-0.6)	n.s.	0.1 (0.0-0.2)

[a] Median absolute count followed by minimal and maximal absolute count in parentheses x 10^9/l;
[b] ncb = neonatal cord blood, 1wk = 1 week of age, 6wk = 6 weeks of age, 3mo = 3 months of age, 6mo = 6 months of age, 1yr = 1 year of age, n.s. = not significant;
[c] also 1wk > 6wk-1yr, 6wk > 3mo-1yr, 3mo > 6mo-1yr, all p < 0.001.

TABLE 3. Absolute counts of CD19+ B-lymphocytes and CD19+ B-lymphocyte subpopulations in eleven infants during follow-up from birth to one year of age and in nine adults.

	neonatal cord blood	1 week of age	6 weeks of age	3 months of age	6 months of age	1 year of age	ncb-1wk < as compared to 6wk-1yr[b]	adults
$CD19^+$ B-lymphocytes	0.8 (0.3-1.0)[a]	0.6 (0.3-1.7)	1.6 (0.9-2.4)	1.7 (1.4-2.0)	1.4 (1.3-2.3)	1.7 (1.2-2.8)	$p < 0.001$	0.2 (0.1-0.4)
B-cell subpopulations:								
$CD37^-/CD20^-/CD19^+$	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.1)	0.0 (0.0-0.1)	0.0 (0.0-0.1)	0.0 (0.0-0.1)	n.s.	0.0 (0.0-0.0)
$CD1c^+/CD19^+$	0.6 (0.3-0.9)	0.5 (0.2-1.6)	1.1 (0.7-1.9)	1.2 (0.9-1.5)	0.9 (0.6-1.3)	0.9 (0.7-1.4)	$p < 0.001$	0.1 (0.0-0.1)
$CD1c^-/CD19^+$	0.4 (0.2-0.9)	0.1 (0.1-0.2)	0.4 (0.2-0.9)	0.5 (0.3-0.7)	0.5 (0.4-1.2)	0.8 (0.4-1.3)	$p < 0.001$	0.2 (0.1-0.2)
$CD5^+/CD19^+$	0.3 (0.1-0.4)	0.3 (0.1-0.8)	0.9 (0.6-1.6)	0.8 (0.6-1.2)	0.7 (0.4-1.2)	0.7 (0.4-1.7)	$p < 0.001$	0.1 (0.0-0.1)
$CD5^-/CD19^+$	0.5 (0.2-0.6)	0.3 (0.1-1.0)	0.7 (0.3-1.1)	0.8 (0.4-1.3)	0.8 (0.5-1.5)	0.8 (0.6-1.8)	n.s.	0.2 (0.1-0.3)
$CD38^+/CD19^+$	0.8 (0.3-1.0)	0.6 (0.3-1.7)	1.5 (0.9-2.3)	1.6 (1.3-2.0)	1.3 (1.2-2.2)	1.6 (1.2-2.6)	$p < 0.001$	0.2 (0.1-0.3)
$CD38^-/CD19^+$	0.0 (0.0-0.0)	0.0 (0.0-0.1)	0.0 (0.0-0.1)	0.1 (0.0-0.1)	0.1 (0.0-0.1)	0.2 (0.0-0.3)	n.s.	0.0 (0.0-0.1)
$CD71^+/CD19^+$	0.0 (0.0-0.4)	0.0 (0.0-0.1)	0.0 (0.0-0.1)	0.1 (0.0-0.1)	0.1 (0.0-0.2)	0.1 (0.0-0.1)	n.s.	0.0 (0.0-0.1)
$CD25^+/CD19^+$	0.0 (0.0-0.1)	0.0 (0.0-0.1)	0.1 (0.0-0.2)	0.1 (0.0-0.1)	0.0 (0.0-0.2)	0.1 (0.0-0.2)	n.s.	0.2 (0.0-0.5)

TABLE 4. Absolute counts of CD16+/56+/CD3- NK-cells and CD16+/56+/CD3- NK-cell subpopulations in eleven infants during follow-up from birth to one year of age and in nine adults.

	neonatal cord blood	1 week of age	6 weeks of age	3 months of age	6 months of age	1 year of age	ncb > as compared to 1wk-1yr[b]	adults
$CD16^+/56^+/CD3^-$ NK-cells	1.0 (0.4-1.6)[a]	0.6 (0.3-1.7)	0.4 (0.2-1.1)	0.5 (0.2-1.3)	0.3 (0.2-1.4)	0.4 (0.3-0.9)	$p < 0.001$	0.2 (0.1-0.4)
NK-cell subpopulations:								
$CD7^+/CD16^+/56^+/CD3^-$	0.6 (0.3-1.2)	0.5 (0.2-1.7)	0.4 (0.2-1.0)	0.4 (0.2-1.3)	0.3 (0.2-1.4)	0.4 (0.3-0.9)	n.s.	0.2 (0.1-0.3)
$CD45RA^+/CD16^+/56^+/CD3^-$	0.6 (0.3-1.2)	0.5 (0.2-1.7)	0.4 (0.2-1.0)	0.4 (0.2-1.3)	0.3 (0.2-1.4)	0.4 (0.2-0.8)	n.s.	0.2 (0.1-0.3)
$CD38^+/CD16^+/56^+/CD3^-$	0.6 (0.2-1.2)	0.5 (0.2-1.7)	0.4 (0.2-1.0)	0.4 (0.2-1.3)	0.3 (0.2-1.4)	0.4 (0.3-0.9)	n.s.	0.2 (0.1-0.3)
$CD45RO^+/CD16^+/56^+/CD3^-$	0.0 (0.0-0.0)	0.0 (0.0-0.1)	0.0 (0.0-0.1)	0.0 (0.0-0.0)	0.0 (0.0-0.1)	0.0 (0.0-0.1)	n.s.	0.0 (0.0-0.0)
$CD8^+/CD16^+/56^+/CD3^-$	0.4 (0.1-0.5)	0.4 (0.1-0.8)	0.2 (0.1-0.3)	0.1 (0.0-0.4)	0.1 (0.0-0.2)	0.1 (0.0-0.2)	$p = 0.001$	0.1 (0.0-0.1)
$CD57^+/CD16^+/56^+/CD3^-$	0.0 (0.0-0.0)	0.0 (0.0-0.1)	0.0 (0.0-0.1)	0.0 (0.0-0.1)	0.0 (0.0-0.2)	0.1 (0.0-0.2)	n.s.	0.1 (0.0-0.2)
$CD25^+/CD16^+/56^+/CD3^-$	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	n.s.	0.0 (0.0-0.0)
$HLA\text{-}DR^+/CD16^+/56^+/CD3^-$	0.0 (0.0-0.2)	0.0 (0.0-0.2)	0.1 (0.0-0.4)	0.0 (0.0-0.1)	0.0 (0.0-0.1)	0.0 (0.0-0.1)	n.s.	0.0 (0.0-0.1)

[a] Median absolute count followed by minimal and maximal absolute count in parentheses x 10^9/l;
[b] ncb = neonatal cord blood, 1wk = 1 week of age, 6wk = 6 weeks of age, 3mo = 3 months of age, 6mo = 6 months of age, 1yr = 1 year of age, n.s. = not significant.

TABLE 5. Relative frequencies of CD3+ T-lymphocytes and CD3+ T-lymphocyte subpopulations in eleven infants during follow-up from birth to one year of age and in nine adults.

	neonatal cord blood	1 week of age	6 weeks of age	3 months of age	6 months of age	1 year of age	adults
$CD3^+$ T-lymphocytes	59 (44-76)[a]	79 (58-89)	71 (61-80)	69 (53-76)	72 (50-78)	65 (50-73)	72 (60-83)
T-cell subpopulations:							
$TCR\alpha\beta^+$	94 (90-97)[b]	97 (94-99)	96 (89-97)	95 (89-97)	95 (90-97)	92 (88-98)	93 (83-97)
$TCR\gamma\delta^+$	3 (1-4)	2 (0-3)	3 (1-9)	3 (1-6)	4 (1-6)	4 (2-8)	5 (2-15)
$CD4^+$	72 (62-82)	76 (69-87)	74 (35-84)	75 (63-86)	56 (34-64)	50 (30-62)	58 (52-69)
$CD8^+$	27 (17-35)	25 (13-31)	25 (15-61)	15 (10-34)	15 (8-32)	16 (8-34)	33 (27-46)
CD4/CD8 ratio	2.7 (1.8-5.0)	2.9 (2.3-6.5)	2.9 (0.6-5.6)	3.5 (1.8-6.6)	3.3 (2.0-7.8)	2.9 (1.8-7.0)	2.0 (1.2-2.7)
$CD4^+/CD8^+$	2 (1-4)	2 (1-5)	2 (1-4)	1 (1-3)	1 (0-3)	1 (0-3)	1 (1-4)
$CD1a^+$	2 (0-7)	1 (0-5)	1 (0-5)	1 (0-4)	0 (0-1)	0 (0-1)	0 (0-2)
$CD45RA^+$	72 (44-94)	79 (52-90)	69 (52-84)	74 (67-91)	80 (64-90)	81 (72-88)	60 (46-71)
$CD45RO^+$	23 (10-76)	26 (16-65)	17 (9-74)	18 (14-50)	15 (13-71)	16 (9-54)	56 (48-71)
$CD38^+$	95 (92-98)	96 (95-98)	95 (91-98)	95 (90-98)	93 (89-96)	90 (87-95)	45 (34-62)
$CD4^+/CD45RA^+$	70 (37-95)	76 (41-88)	65 (57-81)	79 (64-96)	82 (72-91)	80 (65-97)	49 (35-70)
$CD8^+/CD45RA^+$	83 (59-99)	84 (67-97)	83 (42-93)	84 (73-99)	89 (84-94)	89 (81-100)	73 (55-80)
$CD4^+/CD45RO^+$	25 (9-83)	26 (17-73)	19 (10-84)	17 (14-62)	15 (13-90)	16 (11-65)	63 (47-81)
$CD8^+/CD45RO^+$	19 (7-58)	16 (5-43)	13 (4-83)	16 (7-34)	13 (5-65)	15 (7-41)	46 (38-59)
$CD38^-/CD45RA^+$	4 (2-7)	3 (1-4)	3 (2-5)	3 (1-6)	4 (2-7)	5 (2-8)	43 (28-64)
$CD38^+/CD45RA^+$	97 (93-98)	98 (96-99)	96 (95-98)	98 (94-98)	97 (93-98)	95 (93-98)	56 (35-72)
$CD38^-/CD45RO^+$	9 (4-19)	8 (5-13)	14 (2-26)	17 (9-29)	23 (14-30)	23 (13-36)	74 (58-83)
$CD38^+/CD45RO^+$	91 (81-94)	90 (87-94)	87 (74-98)	83 (71-92)	77 (71-85)	77 (64-93)	28 (16-42)
$CD38^-/CD4^+$	2 (1-5)	2 (1-4)	3 (2-6)	3 (2-5)	3 (2-5)	4 (2-7)	27 (17-33)
$CD38^+/CD4^+$	98 (94-99)	98 (94-99)	96 (93-98)	96 (92-98)	96 (92-97)	94 (90-96)	52 (42-75)
$CD38^-/CD8^+$	2 (1-4)	1 (1-3)	1 (1-3)	2 (0-7)	2 (0-4)	4 (1-7)	26 (15-33)
$CD38^+/CD8^+$	93 (90-97)	96 (92-97)	95 (90-99)	93 (85-97)	92 (84-97)	83 (77-95)	32 (21-54)
$CD25^+$	10 (6-15)	13 (10-21)	9 (8-12)	9 (8-11)	9 (6-11)	8 (6-10)	27 (19-36)
HLA-DR^+	9 (1-30)	3 (1-27)	4 (2-70)	3 (2-31)	3 (2-6)	3 (2-7)	28 (3-45)
$CD57^+$	0 (0-1)	0 (0-1)	1 (0-8)	1 (0-12)	1 (0-8)	2 (1-14)	7 (2-17)

[a] Median relative frequency followed by minimal and maximal relative frequency between parentheses within the lymphocyte population;
[b] % positivity of the $CD3^+$ T-lymphocyte subpopulation followed by minimal and maximal values between parentheses.

TABLE 6. Relative frequencies of CD19+ B-lymphocytes, and CD19+ B-lymphocyte subpopulations in eleven infants during follow-up from birth to one year of age and in nine adults.

	neonatal cord blood	1 week of age	6 weeks of age	3 months of age	6 months of age	1 year of age	adults
$CD19^+$ B-lymphocytes	16 (9-21)[a]	10 (6-19)	22 (16-29)	23 (18-28)	22 (18-31)	28 (19-36)	12 (7-18)
B-cell subpopulation:							
$CD37^-CD20^-$	1 (0-2)[b]	2 (1-7)	1 (1-6)	1 (0-3)	1 (1-4)	2 (0-8)	1 (1-3)
$CD5^+$	38 (25-62)	47 (40-67)	62 (48-71)	45 (34-73)	43 (30-68)	48 (29-68)	23 (13-30)
$CD1c^+$	85 (59-92)	87 (72-90)	75 (54-83)	69 (61-79)	64 (46-72)	55 (33-63)	25 (21-29)
$CD38^+$	99 (98-100)	98 (90-99)	98 (95-99)	97 (94-99)	94 (91-97)	93 (82-97)	82 (62-86)
$CD71^+$	7 (2-45)	9 (1-17)	2 (1-6)	3 (1-5)	3 (1-17)	3 (2-7)	8 (3-27)
$CD25^+$	2 (1-29)	6 (2-24)	4 (2-9)	3 (1-8)	3 (1-10)	4 (1-11)	13 (8-26)

TABLE 7. Relative frequencies of CD16+/56+/CD3- NK-cells, and CD16+/56+/CD3- NK-cell subpopulations in eleven infants during follow-up from birth to one year of age and in nine adults.

	neonatal cord blood	1 week of age	6 weeks of age	3 months of age	6 months of age	1 year of age	adults
$CD16^+/56^+/CD3^-$ NK-cells	24 (9-33)[a]	6 (3-9)	6 (3-9)	6 (3-18)	5 (3-19)	6 (3-11)	14 (4-18)
NK-cell subpopulation:							
$CD7^+$	79 (38-93)[b]	94 (82-97)	94 (82-97)	93 (85-98)	91 (84-99)	93 (87-99)	93 (85-97)
$CD45RA^+$	79 (40-92)	94 (86-99)	94 (86-99)	95 (92-99)	95 (83-99)	95 (87-99)	96 (93-98)
$CD38^+$	78 (38-90)	93 (84-98)	93 (84-98)	95 (86-99)	95 (81-99)	95 (87-99)	92 (79-96)
$CD45RO^+$	0 (0-3)	6 (1-12)	6 (1-12)	4 (2-7)	7 (3-14)	5 (2-12)	2 (0-17)
$CD8^+$	37 (19-63)	36 (16-72)	36 (16-72)	36 (6-52)	26 (4-58)	19 (5-62)	29 (11-44)
$CD57^+$	0 (0-2)	0 (0-5)	0 (0-5)	0 (0-24)	12 (0-31)	20 (9-38)	47 (20-58)
$CD25^+$	1 (0-3)	3 (2-7)	3 (2-7)	4 (1-6)	4 (0-6)	1 (1-7)	5 (2-10)
$HLA\text{-}DR^+$	2 (1-18)	14 (4-40)	14 (4-40)	12 (2-22)	10 (2-22)	8 (3-17)	10 (4-21)

[a]Median relative frequency followed by minimal and maximal relative frequency between parentheses within the lymphocyte population;
[b]% positivity within the $CD16^+/56^+/CD3^-$ NK-cell subpopulation followed by minimal and maximal values between parentheses.

"memory" lymphocytes. It is to be expected that these maturational processes are to some extent reflected by changes in the composition of lymphocyte subpopulations during infancy.

Reference values for lymphocyte subpopulations obtained in cross-sectional studies may reflect this gradual process, but changes in the composition of lymphocyte subpopulations in individual children will not occur at exactly the same time-points in life, and will therefore be leveled off in such studies. Longitudinal studies in individual children are more informative about the pattern of lymphocyte subpopulation development in time, and about the influences of external factors like infections and vaccinations upon this development. Also, children with immune disorders are generally followed longitudinally. Therefore, we performed a longitudinal survey of lymphocyte subpopulations in eleven healthy infants during the first year of life and compared these results with those obtained in nine adults. We did not find a significant relation between infection or vaccination and the distribution of lymphocyte subpopulations - except for one time-point - in this small group of eleven healthy infants, however.

We found that T- and B-lymphocyte counts were high throughout the first year of life, with an increase at 1 and 6 weeks of age, respectively. In contrast, NK-cell counts were high at birth and declined after the first week of life, but remained at levels of about twice those found in adults. This is in line with previous data obtained in a cross-sectional study, as we reported recently.[20]

Because of these high lymphocyte counts in infants, differences in subpopulations of T- and B-lymphocytes and NK-cells between infants and adults are better reflected by comparison of their absolute counts than by comparison of their relative frequencies. This is illustrated by our findings in T-lymphocyte, B-lymphocyte, as well as NK-cell subpopulations.

CD4+ as well as CD8+ T-lymphocyte counts followed the pattern of total T-lymphocytes with an increase at 1 week of age and high numbers - especially of CD4+/CD3+ T-lymphocytes - throughout the first year of life. These were mainly CD45RA+ "naive" T-lymphocytes, probably new T-cells, freshly released from the thymus.[21] The absolute counts of CD45RO+ "memory" T-lymphocytes in infants during the first year of life and in adults were *similar.* So, the profound differences in relative frequencies of CD45RA+ "naive" and CD45RO+ "memory" T-lymphocytes between infants and adults are *not* due to changes in the size of the CD45RO+ "memory" pool, but to changes in the size of the CD45RA+ "naive" pool of T-lymphocytes: a large pool of naive "untriggered" cells is present at birth, and increases further during the first year of life, ready for participation in primary immune responses.

Then how is the building of a functional "memory" pool of T-lymphocytes reflected in the distribution of T-lymphocyte subpopulations? Firstly, adult T-lymphocytes probably carried more CD45RO molecules on their surface than infant T-lymphocytes. Secondly, the distribution of the expression of CD38 on

CD45RO+ "memory" (and CD45RA+ "naive") T-lymphocytes was markedly different between infants and adults: it decreased from birth to adulthood, as was described before on total T-lymphocytes.[4,22-28] CD38 is a differentiation lineage-unrestricted cell surface molecule with a role in cellular adhesion, activation and proliferation, which is known to be expressed on thymocytes, on activated peripheral blood T- and B-lymphocytes, and on plasma cells. It is generally regarded as an activation marker.[29] However, we and others[4,28,30,31] did not find other markers of activation in neonatal cord or infant blood, like increased expression of CD25, HLA-DR, CD69, and CD154 (CD40 ligand). Nor did we find an increase of "thymocyte-like" CD1a+, or CD4+/CD8+ "double-positive" T-cells. We therefore presume that the abundant expression of CD38 on infant's T-lymphocytes might be related to a greater metabolic need of the large population of naive "untriggered" cells, due to their continually required participation in primary immune responses.

Little is known about markers expressed by naive "untriggered" B-lymphocytes. Cell surface expression of CD45 isoforms is not informative in B-lymphocytes: all B-lymphocytes express the CD45RA isoform,[32] not the CD45RO isoform.[33] We and others[17] found higher relative frequencies of CD1c+, CD5+, and CD38+ B-lymphocytes in neonates and infants than in adults. The function of CD1c on the surface of B-lymphocytes has not yet been elucidated. CD5+ B-lymphocytes spontaneously produce low-affinity polyreactive IgM autoantibodies, use unmutated V_H genes, and are presumed to represent a more primitive lineage than CD5- B-lymphocytes.[34] We found that CD1c+ and CD5+ B-lymphocyte counts were indeed higher at birth than in adults; they increased even further during the first year of life. CD1c- and CD5- B-lymphocyte counts also increased during the first year of life, albeit not statistically significant for CD5- B-lymphocyte counts. However, the relative frequencies of CD1c+ and CD5+ B-lymphocytes were considerably higher throughout the first year of life than in adults. Therefore, CD1c and CD5 could be markers of "untriggered" B-lymphocytes. CD38 is associated with the CD21/CD19 complex on the surface of B-lymphocytes, and probably has a role as mediator of extracellular signals.[35] The relative frequency of CD38+ B-lymphocytes was higher in infants than in adults. CD38- B-lymphocyte absolute counts were low in infants as well as adults; the high B-lymphocyte counts throughout the first year of life were due to high CD38+ B-lymphocyte counts. The abundant expression of CD38 on infant's B-lymphocytes could be related to a greater metabolic need caused by the many encounters with antigen in primary immune responses, analogous to the situation in T-lymphocytes.

The high NK-cell counts at birth were for a large part accounted for by CD8+ NK-cells. Interestingly, at birth some infants showed large subpopulations of CD7-, CD38-, and CD45RA- NK-cells. It could be that these findings are associated with the maturational status of NK-cells. However, it is more likely that the high absolute counts of NK-cells observed directly after birth are due to a state of NK-cell activation during pregnancy, which rapidly disappears during the first weeks after birth; these activated NK-cells are postulated to represent a fetal 'tumor response' to maternal antigen, or to play a role in placentation.[36] This fits in with the absence

with the absence of CD57+ NK-cells in the first months of life: CD57 is rapidly downregulated upon activation. CD7 is expressed on all resting NK-cells, and is known to be downregulated upon activation.[19] However, we did not find an increase in the number of NK-cells positive for other activation markers at birth. CD8 is involved in the recognition of HLA class I molecules, which are expressed by all nucleated cells. It is intriguing that CD8+ NK-cells are present in such large numbers at birth. Perhaps this is related to the maintenance of pregnancy at the immunological interface between the fetus and its mother. Interestingly, the two infants born at the youngest gestational age (infant nr.6 at 37 weeks, and infant nr.10 at 35 weeks) showed the highest relative frequencies and absolute counts of CD38- NK-cells, CD45RA- NK-cells, and CD7- NK-cells at birth and at 1 week of age. CD8+ NK-cell absolute counts were not higher in these two youngest children, however.

In conclusion, we showed that a detailed longitudinal analysis of the absolute counts of lymphocyte subpopulations in infants contributes to the understanding of lymphocyte development after birth. We found that a large pool of "naive" CD45RA+ - mainly CD4+/CD3+ - T-lymphocytes continues to be produced during the first year of life, ready to be primed in primary immune responses. With priming, further functional maturation is accompanied by changes in the distribution of lymphocyte subpopulations: adult "memory" CD45RO+ T-lymphocytes mostly express no CD38 and probably carry more CD45RO molecules upon their surface. CD1c, CD5 and CD38 are possible markers of "untriggered" B-lymphocytes. NK-cells seem to be more important during pregnancy than thereafter, with absolute numbers falling immediately after birth.

REFERENCES

1 Comans-Bitter WM, de Groot R, van den Beemd R, Neijens HJ, Hop WCJ, Groeneveld K, Hooijkaas H, van Dongen JJM. Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. *J Pediatr* 1997;130:388-393.

2 Denny T, Yogev R, Gelman R, Skuza C, Oleske J, Chadwick E, Cheng S, Connor E. Lymphocyte subsets in healthy children during the first 5 years of life. *JAMA* 1992;267:1484-1488.

3 Erkeller-Yuksel FM, Deneys V, Yuksel B, Hannet I, Hulstaert F, Hamilton C, Mackinnon H, Turner Stokes L, Munhyeshuli V, Vanlangendonck F, deBruyère M, Bach BA, Lydyard PM. Age-related changes in human blood lymphocyte subpopulations. *J Pediatr* 1991;120:216-222.

4 Heldrup J, Kalm O, Prellner K. Blood T and B lymphocyte subpopulations in healthy infants and children. *Acta Paediatr* 1992;81:125-132.

5 Kotylo PK, Fineberg NS, Freeman KS, Redmond NL, Charland C. Reference ranges for lymphocyte subsets in pediatric patients. *Am J Clin Pathol* 1993;100:111-115.

6 Oski F. The erythrocyte and its disorders. In: Nathan D, Oski F (eds) *Hematology of infancy and childhood.* Saunders, Philadelphia, 1993, pp 18-43.

7 Remy N, Oberreit M, Thoenes G, Wahn U. Lymphocyte subsets in whole blood and isolated mononuclear leukocytes of healthy infants and children. *Eur J Pediatr* 1991;150:230-233.

8 Kutvirt SG, Lewis SL, Simon TL. Lymphocyte phenotypes in infants are altered by separation of blood on density gradients. *Br J Biomed Sci* 1993;50:321-328.

9 De Vries E, De Groot R, De Bruin-Versteeg S, Comans-Bitter WM, Van Dongen JJM. Analyzing the developing lymphocyte system of neonates and infants. *Eur J Pediatr* 1999;in press.

10 De Vries E, De Bruin-Versteeg S, Comans-Bitter WM, De Groot R, Boerma GJM, Lotgering FK, Van Dongen JJM. Microassay for immunophenotyping of neonatal lymphocytes with correction for erythroid cell contamination. *Arch Dis Child* 1999;in press.

11 Ezine S, Ceredig R. Haemopoiesis and early T-cell differentiation. *Immunol Today* 1994;15:151-154.
12 Res P, Blom B, Hori T, Weijer K, Spits H. Downregulation of CD1 marks acquisition of functional maturation of human thymocytes and defines a control point in late stages of human T cell development. *J Exp Med* 1997;185:141-151.
13 Aldhous MC, Raab GM, Doherty KV, Mok JYQ, Bird AG, Froebel KS. Age-related ranges of memory, activation, and cytotoxic markers on CD4 and CD8 cells in children. J Clin Immunol 1994;14:289-298.
14 Gratama JW, Vogel EJ, van 't Veer MB, de Gast GC, Bolhuis RLH. Activation of CD57+ lymphocytes in relation to cytomegalovirus infection in healthy carriers, allognenic bone marrow transplant recipients, and B-cell chronic lymphocytic leukaemia. In: Schlossman SF, Boumsell L, Gilks W, Harlan JM, Kishimoto T, Morimoto C, Ritz J, Shaw S, Silverstein R, Springer T, Tedder TF, Todd RF (eds) *Leukocyte typing V. White cell differentiation antigens.* Proceedings of the fifth international workshop and conference held in Boston, USA 3-7 November, 1993. Oxford University Press, Oxford,1995, pp 422-424.
15 Burrows PD, Cooper MD. B cell development and differentiation. *Curr Opin Immunol* 1997;9:239-244.
16 Tsudo M, Uchiyama T, Uchino H. Expression of Tac antigen on activated normal human B cells. *J Exp Med* 1984;160:612-617.
17 Durandy A, Thuillier L, Forveille M, Fischer A. Phenotypic and functional characteristics of human newborns' B lymphocytes. *J Immunol* 1990;144:60-65.
18 Robertson MJ, Cochran KJ, Ritz J. Characterization of surface antigens expressed by normal and neoplastic human NK cells. In: *Leukocyte typing V*, Oxford University Press, 1995, pp.1374-1377.
19 Rabinowich H, Pricop L, Herberman RB, Whiteside TL. Expression and function of CD7 molecule on human natural killer cells. *J Immunol* 1994;152:517-526.
20 De Vries E, de Bruin-Versteeg S, Comans-Bitter WM, de Groot R, Boerma GJM, Lotgering FK, van Dongen JJM. Longitudinal follow up of blood lymphocyte subpopulations from birth to one year of age. *J Pediatr* 1998;133:586-588.
21 Vanhecke D, Leclercq G, Plum J, Vandekerckhove B. Characterization of distinct stages during the differentiation of human CD69+CD3+ thymocytes and identification of thymic emigrants. *J Immunol* 1995;155:1862-1872.
22 Gerli R, Rambotti P, Cernetti C, Velardi A, Spinozzi F. Evidence for phenotypic T precursor cells in human cord blood. *Br J Hematol* 1983;53:685.
23 Foa R, Giubellino MC, Fierro MT, Lusso P, Ferrando ML. Immature T lymphoyctes in human cord blood identified by monoclonal antibodies: a model for the study of the differentiation pathway of T cells in humans. *Cell Immunol* 1984;89:194-201.
24 Griffiths-Chu S, Patterson JAK, Berger CL, Edelson RL, Chu AC. Characterization of immature T cell subpopulations in neonatal blood. *Blood* 1984;64:296-300.
25 Solinger AM. Immature T lymphocytes in human neonatal blood. *Cell Immunol* 1985;92:115-122.
26 Vitiello A, Maccario R, Montagna D, Porta FA, Alberini CM, Mingrat G, Astaldi-Ricotti GCB, Nespoli L, Ugazio AG. Lymphocyte subpopulations in the neonate: a subset of HNK-1-, OKT3-, OKT8+ lymphocytes displays natural killer activity. *Cell Immunol* 1984;85:252-257.
27 DeWaele M, Foulon W, Renmans W, Segers E., Smet L, Jochmans K, van Camp B. Hematologic values and lymphocyte subsets in fetal blood. *AJCP* 1988;89:742-746.
28 Beck R, Lam-Po-Tang PRL. Comparison of cord blood and adult blood lymphocyte normal ranges: A possible explanation for decreased severity of graft versus host disease after cord blood transplantation. *Immunol Cell Biol* 1994;72:440-444.
29 Malavasi F, Funaro A, Roggero S, Horenstein A, Calosso L, Mehta K. Human CD38: a glycoprotein in search of a function. *Immunol Today* 1994;15:95-97.
30 Brugnoni D, Airo P, Graf D, Marconi M, Lebowitz M, Plebani A, Giliani S, Malacarne F, Cattaneo R, Ugazio AG, Albertini A, Kroczek RA, Notarangelo LD. Ineffective expression of CD40 ligand on cord blood T cells may contribute to poor immunoglobulin production in the newborn. *Eur J Immunol* 1994;24:1919-1924.
31 Fulelhan R, Ahern D, Geha RS. Decreased expression of the ligand for CD40 in newborn lymphocytes. *Eur J Immunol* 1994;24:1925-1928.
32 Kupper H, Seifert M, Fiebig H. Characterization of CD45 antibodies and other studies on the use of workshop antibodies for identifying different leukocytes in tissue specimens. In: *Leukocyte typing III,*

McMichael AJ (ed), Oxford University Press, 1987;p.409.
33 Smith SH, Brown MH, Rowe D, Callard RE, Beverley PCL. Functional subsets of human helper-inducer cells defined by a new monoclonal antibody, UCHL1. *Immunology* 1986;58:63-70.
34 Herzenberg LA, Kantor AB, Herzenberg LA.. Layered evolution in the immune system. A model for the ontogeny and develoment of multiple lymphocyte lineages. In: CD5 B cells in development and disease. Proceedings of a conference. *Ann NY Acad Sci* 1991;651:1-9.
35 Funaro A, De Monte LB, Dianzani U, Forni M, Malavasi F. Human CD38 is associated to distinct molecules which mediate transmembrane signaling in different lineages. *Eur J Immunol* 1993;23:2407-2411.
36 Thilaganathan B, Abbas A, Nicolaides KH. Fetal blood natural killer cells in human pregnancy. *Fetal Diagn Ther* 1993;8:149-153.

PART III

LYMPHOCYTE SUBPOPULATIONS IN IMMUNODEFICIENT CHILDREN

CHAPTER 8

IMMUNOPHENOTYPING IN THE DIAGNOSIS AND FOLLOW-UP OF IMMUNODEFICIENT CHILDREN

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Submitted

ABSTRACT

Children with recurrent infections form a large group of patients in pediatric practice. This frequently concerns ear-nose-throat infections and lower respiratory tract infections. Follow-up of these children shows that most of them have local obstructive problems, bronchial hyperreactivity or an allergic constitution. However, some of them might have a primary immunodeficiency, which needs a prompt diagnosis before serious infections compromise the child's general condition.

We developed a multistage diagnostic protocol which allows early exclusion of immunodeficiency diseases by liberal use of screening tests in the first stage, whereas more elaborate tests are reserved for those children where an immunodeficiency disease becomes a more likely diagnosis.

Particularly, flow cytometric immunophenotyping of leukocytes appears to be an efficient and rapid tool in the diagnosis and follow-up of immunodeficient patients.

INTRODUCTION

Immunocompromised children form an increasing group of patients in pediatric practice due to the expanding use of immunosuppressive drugs and aggressive chemotherapy as part of oncological treatment regimens, in autoimmune diseases, or after organ transplantation, as well as due to the increasing prevalence of HIV-infection.[1] From time to time, pediatricians are also confronted with children suffering from primary immunodeficiency diseases.[12] These children should be discerned from the relatively large group of children with recurrent ear-nose-throat (ENT) infections and lower respiratory tract infections due to other causes, such as local obstructive problems, bronchial hyperreactivity, or an allergic constitution.

Laboratory studies for identification and clinical follow-up of immunodeficient children can now be performed in many hospitals. On the one hand, this enables prompt diagnosis of immunodeficiency before serious infections have compromised the child's general condition, reducing the chances of survival.[12] On the other hand, the easy availability of most laboratory tests may lead to excessive use of these expensive tests. The application of a multistage diagnostic protocol allows early exclusion (or identification) of an immunodeficiency due to a low threshold for the performance of simple screening tests in the first stages, whereas more elaborate tests can be reserved for later stages, leading to diagnosis and definitive classification of those cases where a primary immunodeficiency becomes more likely.

In this review, we propose such a multistage diagnostic protocol and focus our discussion on the increasingly important role of flow cytometric

immunophenotyping of leukocytes in the diagnosis and follow-up of immunocompromised children in pediatric practice.[4] Flow cytometric immunophenotyping allows precise assessment of relative frequencies of leukocyte subpopulations by detection of cell surface and intracellular markers with fluorochrome-labeled monoclonal antibodies (McAb). It can be performed within the population of lymphocytes, monocytes, or granulocytes, which are defined on the basis of their light scatter characteristics, such as forward scatter (FSC) as measure for size, and side scatter (SSC) as measure for cellular irregularity (Figure 1).[4] Leukocyte count and differential cell count are then used to calculate absolute counts, which can be compared with age-matched reference values.[2]

DIAGNOSING CHILDREN WITH PRIMARY IMMUNODEFICIENCY

Three distinct clinical categories of primary immunodeficiency diseases can be recognized: antibody deficiencies, cell-mediated or combined (cell-mediated ánd antibody) immunodeficiencies, and granulocyte/monocyte function deficiencies. Children with antibody deficiency usually suffer from recurrent ENT and lower respiratory tract infections. Children with cell-mediated or combined immunodeficiency often suffer from failure to thrive, intractable diarrhea, eczema, and other chronic problems, or they present with an opportunistic infection. Children with granulocyte/monocyte function deficiency may show superficial and systemic infections with pyogenic bacteria or fungi.

It is practical to analyse the possible presence of these different clinical categories of primary immunodeficiency diseases along separate pathways, as is shown in the multistage diagnostic protocol in Table 1. A multistage immunophenotyping protocol with "limited" and "extended" levels is shown in Table 2. The major types of primary immunodeficiency diseases and the characteristic immunophenotyping results found in patients with these diseases are summarized in Table 3.

Children with recurrent ENT and lower respiratory tract infections

Most children with recurrent ENT and lower respiratory tract infections do not have an antibody deficiency, but suffer from local obstructive problems, bronchial hyperreactivity, or an allergic constitution. If these are ruled out, or if infections persist despite adequate treatment of the underlying problem, the presence of a relatively mild antibody deficiency like IgA deficiency, IgG subclass deficiency, or anti-polysaccharide antibody deficiency should be evaluated.[11,12]

It is useful to perform immunophenotyping of lymphocytes according to the "limited" protocol in Table 2 in the following cases:

- severe antibody deficiency with agammaglobulinemia, as found in X-linked agammaglobulinemia (XLA),[12] Igμ deficiency,[16] or λ14.1 deficiency;[10]
- progressive hypogammaglobulinemia with combinations of IgA deficiency,

TABLE 1. Multistage protocol for the exclusion or diagnosis of primary immunodeficiency diseases.

Suspected antibody deficiency in children with recurrent ENT and lower respiratory tract infections

If common underlying problems are ruled out or adequately treated and infections persist:
1. Serum IgG, IgA, IgM and IgG subclass concentrations
2. Specific antibody titers before and after tetanus toxoid and pneumococcal vaccination

In cases of severe antibody deficiency:
3. Immunophenotyping: total blood B-lymphocytes and T-lymphocytes ("limited" protocol)
4. Immunophenotyping: B-cell development in bone marrow, CD40L expression on activated T-lymphocytes ("extended" protocol, specialized/research protocols)
5. Determination of the genetic defect, if applicable

Suspected cell-mediated or combined immunodeficiency in children with failure to thrive, intractable diarrhea, eczema and other chronic problems or opportunistic infections

First diagnostic work-up:
1. Leukocyte count and differential; serum IgG, IgA and IgM concentrations; HIV serology and HIV PCR
2. Immunophenotyping: the major lymphocyte populations ("limited" protocol); *N.B.* confounding presence of maternal T-lymphocytes might occur in SCID (tests in specialized laboratories are then required)

If abnormalities have been detected, or immunodeficiency is highly suspected:
3. Lymphocyte proliferation *in vitro* after stimulation with mitogens
4. Immunophenotyping: focussing on specific diseases ("extended" protocol, specialized/research protocols)
5. Specific measurements for certain diseases (e.g. ADA and PNP enzyme levels, karyotype and DNA-repair, α-foetoprotein for AT, skeletal X-rays for cartilage hair dysplasia)
6. More elaborate proliferation protocols (*in vitro* lymphocyte proliferation after stimulation with McAb or antigens; *in vivo* delayed hypersensitivity via skin tests)
7. Determination of the genetic defect, if applicable

Suspected granulocyte/monocyte function deficiency in children with superficial and systemic infections with pyogenic bacteria or fungi

First diagnostic work-up:
1. Leukocyte count and differential
2. Hematological assessment if indicated
3. Consider antibody deficiency, or cell-mediated or combined immunodeficiency (see above)

If other causes are excluded and the infections persist:
4. Granulocyte function testing (chemotaxis, phagocytosis, intracellular killing of bacteria)
5. Immunophenotyping in case of granulocytosis: CD18 and CD15s expression ("extended" protocol)
5. Determination of the genetic defect, if applicable

Abbreviations: ADA = Adenosine deaminase deficiency, AT = ataxia teleangiectasia, HIV = human immunodeficiency virus, McAb = monoclonal antibody, PCR = polymerase chain reaction, PNP = purine nucleotide phosphorylase deficiency.

IgG subclass deficiency, and/or anti-polysaccharide antibody deficiency, as found in common variable immunodeficiency (CVID);[12,14]
- severe hypogammaglobulinemia with normal or increased IgM, as found in X-linked hyper IgM syndrome (XHIM).[7]

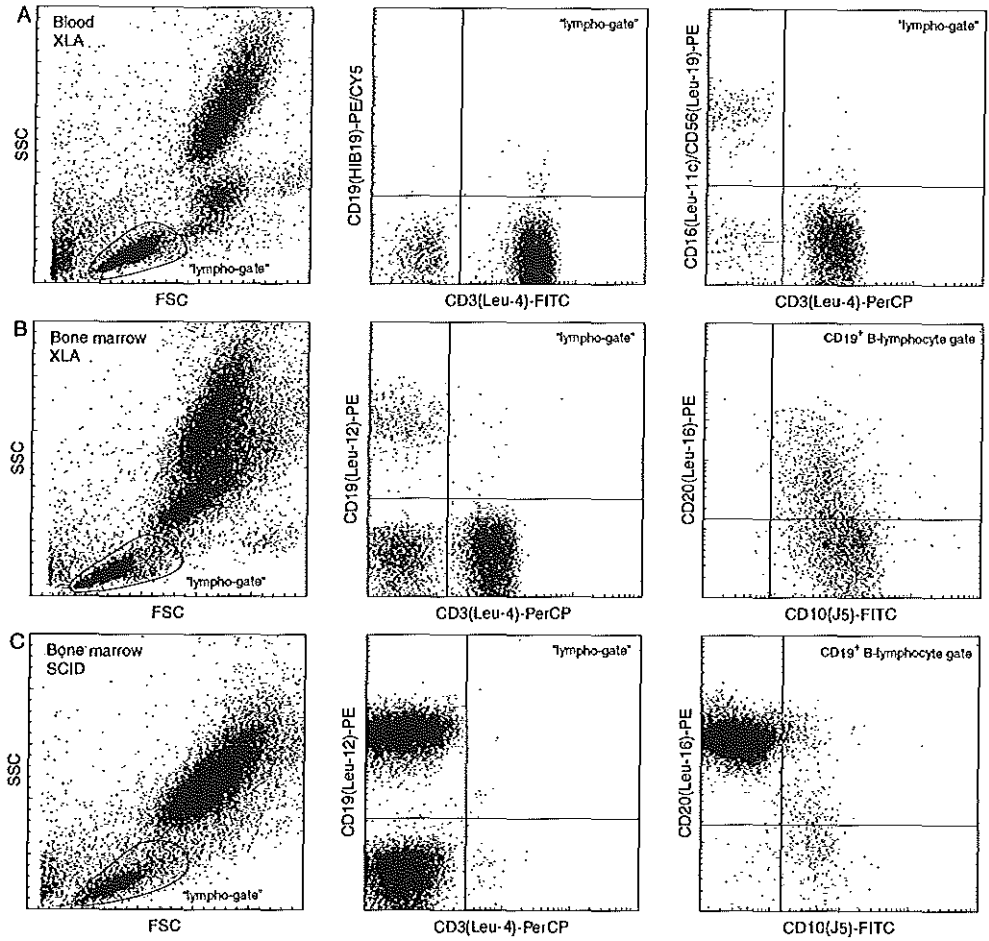

Figure 1. Flow cytometric immunophenotyping of lymphocytes in blood and bone marrow of immunodeficient patients. *A*, Analysis of peripheral blood of an XLA-patient. Dot plot with FSC and SSC showing "lympho-gate" (left). Absence of CD19+ B-lymphocytes (middle), with CD3+ T-lymphocytes and CD16+ and/or CD56+/CD3- NK-cells normally present (right). *B*, Analysis of bone marrow of an XLA-patient. Dot plot with FSC and SSC showing "lympho-gate" (left). Very few CD19+ B-lineage cells are present (middle), which are mostly CD10+/CD20-/CD19+ precursor-B-cells (right). *C*, Analysis of bone marrow of a SCID patient. Dot plot with FSC and SSC showing "lympho-gate" (left). Absence of CD3+ T-lymphocytes (middle), leading to a relatively predominant presence of mature CD10-/CD20+/CD19+ B-lymphocytes within the "lympho-gate".

If immunophenotyping of lymphocytes shows that B-lymphocytes are absent in the peripheral blood compartment (Figure 1a), this strongly suggests a diagnosis of XLA or - less frequently - Igμ deficiency. Analysis of the maturational arrest by morphologic and immunophenotypic studies of bone marrow cells with an extensive research protocol can then direct the diagnostic analysis at the

TABLE 2. Flow cytometric immunophenotyping protocol for the diagnosis of primary immunodeficiency diseases in pediatric practice.

"Limited" protocol (use absolute counts and age-matched reference values)
T-lymphocytes: total CD3+ CD4+/CD3+ helper subset CD8+/CD3+ suppressor/cytotoxic subset
B-lymphocytes: total CD19+ or CD20+
NK-cells: defined as CD3- as well as CD16+ and/or CD56+ population
"Extended" protocol (compare with normal age-matched controls run in parallel if no reference values are available)
CD40L expression on activated T-lymphocytes if XHIM is suspected
HLA-DR (class II) and β_2 microglobulin (class I) expression on leukocytes if BLS is suspected
TCR$\alpha\beta$ and TCR$\gamma\delta$ expression on T-lymphocytes if CATCH-22 is suspected
CD18 and CD15s expression on leukocytes if LAD1 or LAD2 is suspected

Abbreviations: BLS = bare lymphocyte syndrome; LAD = leukocyte adhesion deficiency; XHIM = X-linked hyper IgM syndrome.

molecular level: XLA results in the arrest of B-cell development in the bone marrow at a later maturational stage than Igμ deficiency (Figures 1b and 2).[9]

If B-lymphocytes are found to be present in the peripheral blood compartment, CVID or XHIM are more likely diagnoses. Immunophenotyping of blood lymphocytes according to the "limited" protocol in Table 2 can show accompanying T-lymphocytopenia in CVID.[14] In XHIM, immunophenotyping of activated blood T-lymphocytes shows absent or decreased expression of CD40 ligand (CD40L) ("extended" protocol in Table 2).[7,12] This absent or decreased expression of CD40L leads to disturbed CD40L-CD40 mediated T-B cell communication, which is indispensable for isotype switching from IgM to IgG, IgA or IgE. The definitive diagnosis of XHIM can be made via mutation analysis of the *CD40L* gene.

Children with failure to thrive, intractable diarrhea, eczema, and other chronic problems, or with an opportunistic infection

Many children with cell-mediated or combined immunodeficiency do not present with recurrent infections, but with failure to thrive, intractable diarrhea, eczema, and other chronic problems.[6] The differential diagnosis is extensive in these cases. The combination of lymphopenia and agammaglobulinemia can direct the search towards an immunodeficiency, but these are not invariably present. Opportunistic infections almost always indicate an immunodeficiency, either acquired (see below) or congenital.

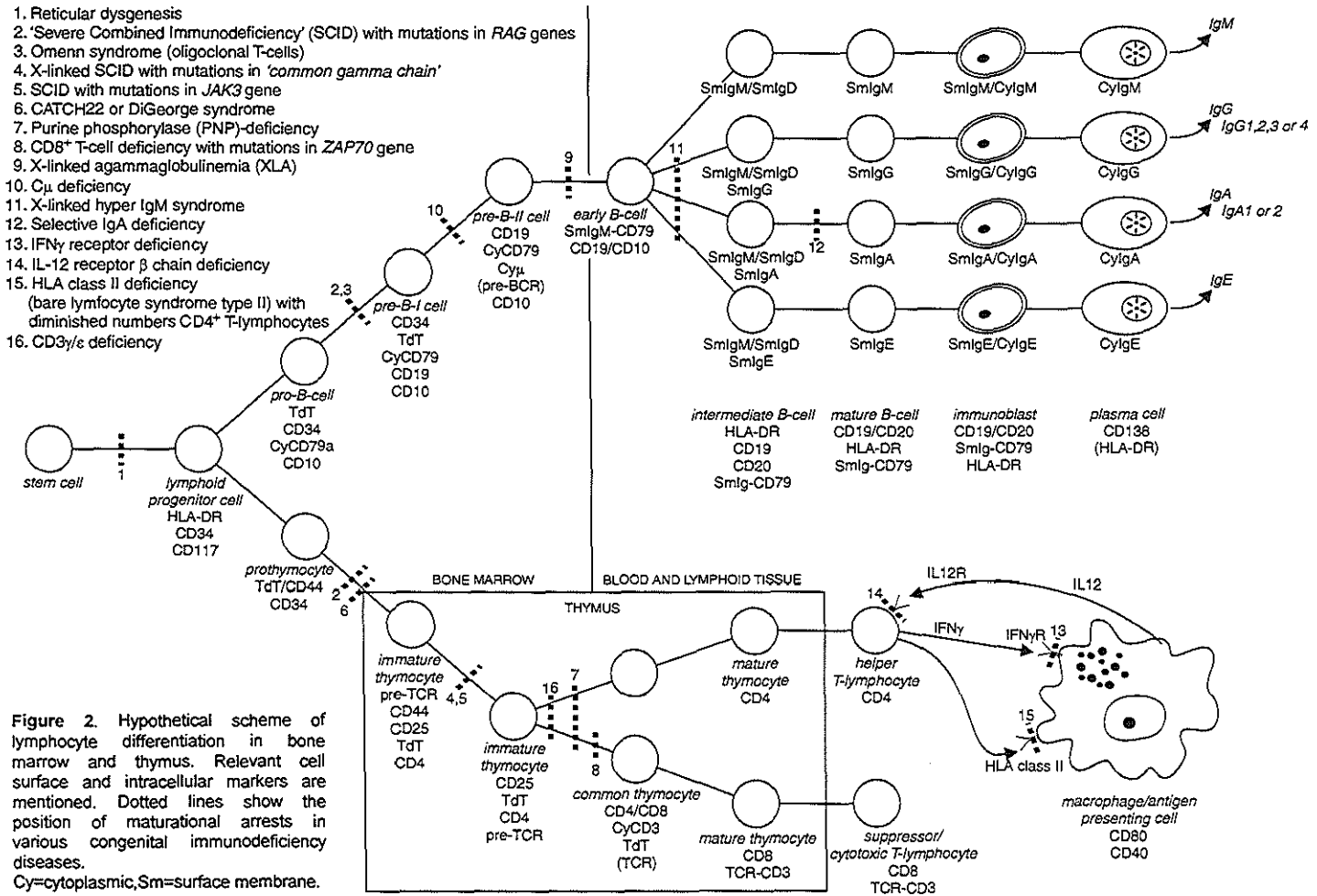

Figure 2. Hypothetical scheme of lymphocyte differentiation in bone marrow and thymus. Relevant cell surface and intracellular markers are mentioned. Dotted lines show the position of maturational arrests in various congenital immunodeficiency diseases.
Cy=cytoplasmic,Sm=surface membrane.

The "limited" immunophenotyping protocol as shown in Table 2 can easily detect severe combined immunodeficiency disease (SCID): T-lymphocytes will be absent or very low in number (Figure 1c). The presence or absence of B-lymphocytes differentiates between B+ SCID and B- SCID, as found in X-linked common γ-chain deficiency (XSCID) or autosomal recessive JAK3 and RAG1 or RAG2 deficiency, respectively (Figure 1c).[6,12] Changes in either the CD4+/CD3+ helper T-lymphocyte count or the CD8+/CD3+ suppressor/cytotoxic T-lymphocyte count can indicate a diagnosis of "bare lymphocyte syndrome" (BLS type II or MHC class-II expression deficiency) with low CD4+/CD3+ counts or a diagnosis of ZAP70 deficiency with low CD8+/CD3+ counts, respectively.[12]

However, in many cases of combined immunodeficiency (CID), like adenosine deaminase (ADA) and purine nucleotide phosphorylase (PNP) deficiency, Wiskott-Aldrich syndrome (WAS) and ataxia teleangiectasia (AT), immunophenotyping results are less aberrant with T-lymphocytopenia developing over time.[12] Additional tests are then required to come to a diagnosis (see Table 1).

If immunodeficiency is highly suspected, but the "limited" immunophenotyping protocol shows no abnormalities, an "extended" immunophenotyping protocol as shown in Table 2 can identify additional children with specific subtypes of cell-mediated or combined immunodeficiency that are not accompanied by major changes in the main lymphocyte populations. For example, in MHC class-II deficiency the absence of HLA-DR surface expression is the definitive clue to the diagnosis;[12] CATCH-22 syndrome is sometimes accompanied by severe T-lymphocytopenia with low to absent TCRαβ+ T-lymphocytes and normal numbers of TCRγδ+ T-lymphocytes (J.J.M. van Dongen, unpublished observation).

In unclassifiable cases, extensive specialized protocols may be needed to unravel the immunodeficiency. These protocols may include the following:[4,5,12]

- HLA-typing or sex-chromosome analysis of individual T-lymphocytes in male patients to detect the maternal origin of T-lymphocytes in SCID patients with maternal graft versus host disease;
- markers of maturation, such as CD45RA and CD45RO;
- markers of activation and/or proliferation, such as CD25, CD69, CD40L and Ki67;
- co-stimulatory and/or signalling molecules, such as CD27⇌ CD70, and CD28 ⇌ CD80/CD86
- intracellular cytokines, such as IL2 and IFNγ (Th1 profile), and IL4, IL5, and IL10 (Th2 profile);
- specialized function tests, such as *in vitro* immunoglobulin production.

These tests are generally not performed in routine laboratories, and will not be further discussed here.

Children with superficial and systemic infections with pyogenic bacteria or fungi

Most children with superficial and systemic infections with pyogenic bacteria or fungi suffer from neutropenia due to hematologic disorders or iatrogenic causes

TABLE 3. Immunophenotyping in the diagnosis of primary immunodeficiency diseases.

DISEASES	PATHOGENESIS	IMMUNOPHENOTYPING
MILD ANTIBODY DEFICIENCIES (Recurrent ENT and lower respiratory tract infections, lambliasis, sometimes asymptomatic)		
- IgA deficiency - IgG subclass deficiency - Anti-polysaccharide antibody deficiency	Pathogenesis unknown	Normal immunophenotyping results
SEVERE ANTIBODY DEFICIENCIES (Recurrent respiratory tract and ENT infections, life-threatening bacterial infections, enteroviral CNS infections, lambliasis)		
- XLA (Btk enzyme deficiency, X-linked) - Igμ deficiency (autosomal recessive) - λ14.1 deficiency (autosomal recessive)	Early arrest in B-cell development	Absent blood B-lymphocytes; maturational arrest reflected in precursor B-cell population in bone marrow
- XHIM	CD40L deficiency leads to absent isotype switch of B-lymphocytes and associated T-lymphocyte defect due to absent CD40 stimulation	Absent or decreased CD40L expression on activated T-lymphocytes; blood B-lymphocytes present
- CVID	Probably defects in B-cell maturation and helper T-lymphocyte function	Blood B-lymphocytes present; sometimes T-lymphocytopenia
COMBINED IMMUNODEFICIENCY DISEASES (Failure to thrive, intractable diarrhea, eczema, and other chronic problems, or opportunistic infections)		
- ADA deficiency (autosomal recessive) - PNP deficiency (autosomal recessive)	Accumulation of toxic purine metabolites due to enzyme deficiency	Increasing T-lymphocytopenia with time; also B-lymphocytopenia in ADA- SCID
- MHC class-II expression deficiency (BLS type II, autosomal recessive)	Impaired antigen presentation	Deficient MHC class-II expression; low numbers of CD4+ T-lymphocytes
- WAS/XLT (X-linked) (patients also suffer from eczema, thrombocytopenia, and malignancy)	Impaired cytoskeletal reorganization of platelets and T-lymphocytes	Increasing T-lymphocytopenia with time; impaired CD43 expression

DISEASE	PATHOGENESIS	IMMUNOPHENOTYPING
SEVERE COMBINED IMMUNODEFICIENCIES		
(Failure to thrive, intractable diarrhea, eczema, and other chronic problems, or opportunistic infections)		
- X-SCID (common γ-chain deficiency, X-linked) - JAK-3 kinase deficiency (autosomal recessive)	Defect in T-lymphocyte and NK-cell development	T-lymphocytes absent, NK-cells low, and B-lymphocytes present
- RAG1 or RAG2 deficiency (autosomal recessive)	Early arrest in common lymphocyte development	T- and B-lymphocytes absent
- Omenn syndrome; some RAG1 deficiencies (autosomal recessive)	Defective T-lymphocyte development with B-lymphocyte deficiency	Oligoclonal T-lymphocytes, very low or absent B-lymphocytes
- ZAP-70 kinase deficiency (autosomal recessive)	Defect in T-lymphocyte development	T-lymphocytes - especially CD8+ decreased; B-lymphocytes present
- CD3 γ- or ε-chain deficiency (autosomal recessive)	Defect in T-lymphocyte function	T-lymphocytes decreased, CD3 expression decreased; B-lymphocytes present
- Reticular dysgenesis	Stem cell defect leading to defective maturation of T- and B-cells and myeloid cells	Pancytopenia
MILDER IMMUNODEFICIENCIES ACCOMPANYING OTHER DISEASES		
- DiGeorge (CATCH-22 syndrome)	Cardiac malformations, hypoparathyroidism	Sometimes TCRαβ+ T-lymphocytopenia with normal TCRγδ+ T-lymphocyte counts
- AT (autosomal recessive)	Progressive ataxia, teleangiectasia	Sometimes increasing T-lymphocytopenia with time
- Chromosomal breakage syndromes (Nijmegen breakage syndrome, Blooms syndrome, ICF syndrome)	Mental retardation, photosensitivity, abnormal physionomy	Immunophenotyping mostly normal

Abbreviations: AT = ataxia teleangiectasia; ADA = adenosine deaminase; BLS = bare lymphocyte syndrome; Btk = Bruton's tyrosine kinase; CNS = central nervous system; CVID = common variable immunodeficiency; ENT = ear-nose-throat; HLA = human leukocyte antigen; ICF = immunodeficiency-centromeric instability-facial dysmorphism; JAK = Janus-associated kinase; MHC = major histocompatibility complex; PNP = purine nucleotide phosphorylase; RAG = recombinase activating gene; WAS = Wiskott Aldrich syndrome; XHIM = X-linked hyper IgM syndrome; XLA = X-linked agammaglobulinemia; XLT = X-linked thrombocytopenia; XSCID = common γ-chain deficiency.

(see below). This is easily detected with a leukocyte count and differential. A defect in granulocyte/monocyte function, such as in chronic granulomatous disease (CGD) or leukocyte adhesion deficiency (LAD), is rare. In CGD, granulocytes show deficient microbial killing due to a defect in one of the five NADPH oxidase complex components. This can be investigated by function tests of granulocytes.[12] In LAD1 (CD18 deficiency) and LAD2 (CD15s deficiency) leukocyte adhesion is impaired. This leads to leukocytosis, which is even more pronounced during infections.[12] CD18 and CD15s expression can be evaluated with immunophenotyping.

FOLLOWING CHILDREN WITH ACQUIRED IMMUNODEFICIENCY

Most cases of acquired immunodeficiency are iatrogenic and can therefore be anticipated: as more and more children are being treated with immunosuppressive drugs, aggressive chemotherapy protocols, and bone marrow transplantation, acquired immunodeficiency becomes a more common problem in pediatric practice.

During follow-up of children treated with immunosuppressive drugs or chemotherapy, the degree of neutropenia is easily assessed with a leukocyte count and differential. The slow reconstitution of lymphocytes after bone marrow transplantation can be monitored by a simple immunophenotyping protocol to determine the numbers of the main lymphocyte populations ("limited" protocol in Table 2). It should be kept in mind, however, that these cells remain functionally deficient for a longer period of time than is needed for the normalization of their absolute counts.[13]

HIV-infection is generally diagnosed by serology or PCR.[15] Immunophenotyping has a role in the follow-up of HIV-infected children with the determination of decreasing CD4+/CD3+ helper T-lymphocyte counts as a prognostic marker for the development of AIDS, and - together with RNA quantification - for the timing of antiretroviral therapy.[15] During antiretroviral therapy monitoring of CD4+ helper T-lymphocyte counts is useful for evaluating the treatment effectiveness in addition to RNA quantification.

More elaborate immunophenotyping protocols can be reserved for research purposes, e.g. for comparison of the immune reconstitution after bone marrow transplantation with normal ontogeny.[13] Such studies are important for understanding the regeneration processes, but have no direct clinical relevance as yet.

CONCLUSION

A multistage diagnostic protocol as shown in Table 1 identifies immunodeficient children in an efficient way: simple tests applied to a larger group

of children with *potential* immunodeficiency allow early diagnosis or exclusion, whereas more elaborate tests are reserved for those few children where immunodeficiency is more *probable*.

Immunophenotyping of leukocytes forms an important part of the multistage protocol: determination of the presence and number of the main lymphocyte populations ("limited" protocol) identifies (S)CID patients and B-lymphocytopenia in agammaglobulinemic patients, and, in selected cases, a set of extra measurements ("extended" protocol) identifies children with immunodeficiencies that are not accompanied by major changes in the main lymphocyte populations (Table 2). However, it should be noted that not all children with an immunodeficiency show alterations in immunophenotyping results.

More extensive immunophenotyping protocols used in specialized laboratories or research settings help to recognize new immunodeficiency diseases by identifying as yet undescribed staining patterns. Also, pathogenesis of known defects can be further elucidated: the absence of specific cell surface markers,[12] the loss of specific epitopes defined by McAb staining patterns,[3,8] or the determination of the B-cell differentiation blockade in bone marrow[9] can support the precise characterization of the defect. Therefore, it is important to use these more extensive immunophenotyping protocols in patients with an established immunodeficiency disease, as well as in patients with probable immunodeficiency disease that has not been proven with other methods.

Due to the rapid developments in the field of flow cytometry and antibody production, it is likely that the "limited" and "extended" immunophenotyping protocols defined here will have to be updated in a few years time.

REFERENCES

1 Cherry JD, Feigin RD. Infection in the compromised host. In: Stiehm ER (ed.). Immunologic disorders in infants & children. Saunders, Philadelphia, 1996, pp 975-1013.
2 Comans-Bitter WM, de Groot R, van den Beemd R, Neijens HJ, Hop WCJ, Groeneveld K, Hooijkaas H, van Dongen JJM. Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. J Pediatr 1997;130:389-393.
3 De Vries E, Koene HR, Vossen JM, Gratama J-W, von dem Borne AEGKr, Waaijer JLM, Haraldsson A, de Haas M, van Tol MJD. Identification of an unusual Fcγ receptor IIIa (CD16) on natural killer cells in a patient with recurrent infections. Blood 1996;88:3022-3027.
4 Fleisher TA. Immunophenotyping of lymphocytes by flowcytometry. Immunol Allergy Clin 1994;14:225-240.
5 Fleisher TA, Tomar RH. Introduction to diagnostic laboratory immunology. JAMA 1997;278:1823-1834.
6 Hong R, Clement T, Gatti RA, Kirkpatrick CH. Disorders of the T-cell system. In: Stiehm ER (ed) Immunologic disorders in infants & children. Saunders, Philadelphia, 1996, pp 339-408.
7 Kroczek RA, Graf D, Brugnoni D, Giliani S, Korthäuer U, Ugazio A, Senger G, Mages HW, Villa A, Notarangelo LD. Defective expression of CD40 ligand on T cells causes "X-linked immunodeficiency with hyper-IgM (HIGM1)". Immunol Rev 1994;138:39-59.
8 Kuijpers TW, van Lier RA, Hamann D, de Boer M, Thung LY, Weening RS, Verhoeven AJ, Roos D. Leukocyte adhesion deficiency type 1 (LAD-1)/variant. A novel immunodeficiency syndrome characterized by dysfunctional beta2 integrins. J Clin Invest 1997;100:1725-1733.

9 Meffre E, LeDeist F, de Saint-Basile G, Deville A, Fougereau M, Fischer A, Schiff C. A non-XLA primary deficiency causes the earliest known defect of B cell differentiation in humans: a comparison with an XLA case. Immunology Letters 1997;57:93-99.
10 Minegishi Y, Coustan-Smith E, Wang Y-H, Cooper MD, Campana D, Conley ME. Mutations in the human *λ5/14.1* gene result in B cell deficiency and agammaglobulinemia. J Exp Med 1998;187:71-77.
11 Ochs HD, Winkelstein J. Disorders of the B-cell system. In: Stiehm ER (ed) Immunologic disorders in infants & children. Saunders, Philadelphia, 1996,pp 296-338.
12 Rosen FS, Wedgwood RJP, Eibl M, Fischer A, Aiuti F, Notarangelo L, Kishimoto T, Resnick IB, Hammarstrom L, Seger R, Chapel H, Thompson RA, Cooper MD, Geha RS, Good RA, Waldmann TA . Primary immunodeficiency diseases. Report of a WHO scientific group. Clin Exp Immunol 1997;109 (S1):1-28.
13 Small TN, Keever CA, Weiner-Fedus S, Heller G, O'Reilly RJ, Flomenberg N. B-cell differentiation following autologous, conventional, or T-cell depleted bone marrow transplantation: a recapitulation of normal B-cell ontogeny. Blood 1990;76:647-1656.
14 Spickett GP, Farrant J, North ME, Zhang J, Morgan L, Webster DB. Common variable immunodeficiency: how many diseases? Immunol Today 1997;18:325-328.
15 Working group on antiretroviral therapy and medical management of infants, children and adolescents with HIV infection. Antiretroviral therapy and medical management of pediatric HIV infection. Pediatrics 1998;102 (S4):1005-1062.
16 Yel L, Minegishi Y, Coustan-Smith E, Buckley RH, Trübel H, Pachman LM, Kitchingman GR, Campana D, Rohrer J, Conley ME. Mutations in the mu heay chain gene in patients with agammaglobulinemia. N Eng J Med 1996;335:1486-1493.

CHAPTER 9

RECONSTITUTION OF LYMPHOCYTE SUBPOPULATIONS AFTER PEDIATRIC BONE MARROW TRANSPLANTATION

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Submitted

ABSTRACT

We prospectively studied the reconstitution of lymphocyte subpopulations in a group of 22 children, who survived disease-free at least 6 months after allogeneic bone marrow transplantation (BMT) for a hematological malignancy.

Absolute counts of lymphocytes, B-lymphocytes, T-lymphocytes, and CD4+ helper T-lymphocytes reached the 5th percentile (p_5) of age-matched reference values within 6 months after BMT in 15, 17, 7, and 2 patients, respectively. Especially CD4+ helper T-lymphocyte reconstitution was very slow. Unexpectedly, CMV-reactivation had a profound positive influence upon the number of CD4+ helper T-lymphocytes in the children. In 5 patients, B-lymphocyte absolute counts above the 95th percentile were reached from 6 months after BMT onwards, mimicking normal ontogeny.

Unlike normal ontogeny, the percentages of helper T-lymphocytes expressing the "naive" CD45RA isoform were low and those expressing the "memory" CD45RO isoform were high in the first 3 months after BMT, as was described before. Thereafter, the CD45RA:CD45RO ratio slowly normalized. Also, CD7 expression was absent on up to 90% of T-lymphocytes in the first months after BMT, and on a steadily decreasing percentage thereafter, as was recently described in adults. However, the *absolute counts* of CD45RO+/CD4+ and CD7-/CD4+ helper T-lymphocytes did not change significantly. So, we found no evidence of peripheral expansion of previously primed donor-derived "memory" T-lymphocytes during the follow-up period of 1-18 months after BMT. The absolute counts of "naive" CD45RA+ helper T-lymphocytes did not show a faster increase after BMT than was described in adults, despite the presumed presence of a non-involuted thymus in children.

INTRODUCTION

Bone marrow transplantation (BMT) is increasingly used as a potential curative therapy for hematological and other malignancies, immunodeficiency diseases, and lately also for metabolic and autoimmune disorders.[1-3] Unfortunately, infectious morbidity and mortality resulting from the period of immunodeficiency early posttransplant are an important side effect of BMT.

Studies on the immune reconstitution after allogeneic BMT have been performed extensively in adults.[4-9] The innate immune system (phagocytes) fully recovers in the first weeks to months after BMT, but complete functional reconstitution of the adaptive immune system (B- and T-lymphocytes) takes much longer. Therefore, infectious complications continue to be a problem for many months after BMT.[3,10] Especially, the reconstitution of CD4+ helper T-lymphocytes is very slow in adults. This could be related to the decreasing thymic function with age. Presumably, reconstitution of the T-cell compartment shortly after BMT is mainly provided by a peripheral expansion of donor T-lymphocytes present in the

graft, and not by donor stem cells undergoing the normal developmental pathways in the bone marrow and thymus.[4,11] This fits in with the observation that CD45RO+ "memory" CD4+ helper T-lymphocytes are present at a high proportion in peripheral blood after BMT in adults.[12] Results of studies on T-cell regeneration after courses of intensive chemotherapy indicate that the thymus-dependent repopulation of "naive" CD4+ helper T-lymphocytes occurs with a shorter delay in children than in adults.[13,14] In children, however, only limited data on immune reconstitution after BMT are available.[15,16]

Therefore, we longitudinally investigated the T- and B-cell lineages in peripheral blood by immunophenotyping in 22 children after allogeneic BMT for a haematological malignancy. Because of the low total lymphocyte counts shortly after BMT, changes in lymphocyte subpopulation size are better represented by their absolute counts than by their relative frequencies.[17] So, we studied the dynamics of reconstitution by calculating the absolute counts of the different lymphocyte subpopulations. These results were related to age-matched reference values for healthy children from the literature if available,[17] and otherwise to the values found in age-matched BMT donors.

MATERIALS AND METHODS

Patients

Between October 1990 and April 1994, absolute counts and relative frequencies of peripheral blood lymphocyte subpopulations were determined prospectively in 22 of 34 consecutive pediatric patients who underwent allogeneic BMT for a hematological malignancy at the Department of Pediatrics of the Leiden University Medical Centre. These 22 children survived disease-free for at least 6 months posttransplant and had at least 3 posttransplant samples analyzed during up to 18 months of follow-up. Clinical data concerning these children are summarized in Table 1.

Surveillance for herpesvirus-reactivation

At weekly intervals after BMT, throat swaps were cultured for herpes simplex virus (HSV), and throat swaps and urine samples were cultured for cytomegalovirus (CMV). White blood cells were investigated once weekly from 3 weeks after BMT onwards for the presence of the nuclear CMV-related antigen pp65 in seropositive recipients.[19] Serology for HSV, varicella zoster virus (VZV), CMV and Epstein-Barr virus (EBV) was performed before BMT, and on indication after BMT (results in Table 1). Reactivation was defined as isolation of the virus from the throat (HSV), the throat and/or urine (CMV) or antigen detection in the blood (CMV). Disease was defined as reactivation combined with signs of organ involvement, e.g. hepatitis, pneumonitis (CMV), mucositis (HSV), skin lesions (VZV), and lymphoproliferation (EBV). Eleven patients were at risk for CMV-related problems (positive serology pre-BMT in donor and/or recipient), 13 for HSV-, 17 for EBV-, and 18 for VZV-related problems (Table 1). None of the patients received viral prophylaxis.

Immunostaining of membrane antigens

Leukocyte counting was performed on EDTA-blood using a Sysmex F800 hematological counter (Toa Medical Electronics, Kobe, Japan), and leukocytes were differentiated on blood smears stained with May-Grünwald Giemsa. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation. Interface cells were resuspended in lysisbuffer to remove the erythrocytes, followed by an incubation at 37 ^{0}C to remove immunoglobulins bound non-specifically to the cell membrane.

The various cell populations present among PBMC were identified according to the surface expression of lineage-specific antigens after two-colour immunostaining (Table 2). CD3+/CD8+ suppressor/cytotoxic T-lymphocytes were not measured directly in the oldest samples (25%), but estimated by the investigation of total CD8+ lymphocytes. In short, 50 µl of the cell suspension containing 0.1-1.0 x 10 ^{6} PBMC was incubated in the dark for 30 minutes at room temperature with 25 µl of fluorochrome-conjugated monoclonal antibodies (MoAbs). The cells were washed once with phosphate buffered saline (PBS) containing 1% (w/v) bovine serum albumin (PBS-BSA). All media contained NaN_3 to prevent antibody internalization.

Immunostaining patterns were analyzed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA), and data were analyzed using the Cellquest software package. A gate was set around the lymphocyte population on the basis of forward and side scatter characteristics and staining patterns of CD45 and CD14 MoAbs (CD45+/CD14- cells).

Recovery of lymphocyte subpopulations

Normalization of the absolute counts of a given lymphocyte (sub)population in the peripheral blood of a patient was defined as reaching the 5th percentile (p_5) of age-matched reference values published in the literature.[17] In case age-matched reference values were not available, a comparison was made with data from age-matched BMT donors analyzed between September 1992 and April 1997 (1-5 years of age, n=29; 5-10 years of age, n=30; 10-18 years of age, n=45).

CMV-specific lymphocyte activation

Child no. 21 suffered from CMV-reactivation with pp65 detectable in white blood cells and CMV cultured from urine from 1 to 4.5 months after BMT. CMV-specific T-cell responses were measured at day +80, day +220, and day +264 posttransplant. Briefly, PBMC were stimulated with CMV (AD169) infected HEL-cells, and uninfected HEL-cells as control. After 6 hours of stimulation, cells were analyzed by flow cytometry for surface expression of the activation marker CD69 and the intracellular presence of interferon (IFN) γ according to Waldrup et al.[20] After 5 days of stimulation ^{3}H-thymidine was added to the cultures and incorporation was measured during a final 16 hours of incubation.

Statistics

The p_5 recovery data were analyzed with life table techniques, taking into

TABLE 1. Patient's characteristics and reconstitution data.

No.	Sex	Age[1]	Donor	TCD	Diagnosis	Conditioning	GvH-prophylaxis	GvHD	CMV[2]			HSV			EBV			VZV			Reconstitution after BMT[4]			
									D	R	CI	D	R	CI	D	R	CI	D	R	CI	T	Th	Ts/c	B
1	M	3.3	HLA-id	-	AML II	TBI/Cy +AraC	CsA/MTX	-	+	+	r	+	-	-	-	+	-	-	+	-	<28	28-77	<28	28-42
2	F	15	HLA-id	-	AML I	TBI/Cy +AraC	CsA/MTX	-	-	-	-	+	+	-	+	-	-	-	-	-	167+	167+	<32	32-90
3	M	8.8	HLA-id	-	AML I	TBI/Cy +AraC	CsA/MTX	-	-	+	r	+	+	-	+	+	-	+	+	-	<28	78-239	<28	29-50
4	F	1.7	HLA-id	-	ALL I	TBI/Cy +VP16	CsA/MTX	-	-	+	-	-	n	-	-	n	-	+	n	-	85-167	358+	85-167	<57
5	M	5.5	HLA-id	-	ALL II	TBI/Cy +VP16	CsA/MTX	-	-	-	-	-	-	-	-	-	-	-	-	-	85-162	162-344	<27	27-58
6	M	13.2	HLA-id	-	ALL II	TBI/Cy +VP16	CsA/MTX	-	+	-	r	-	+	-	-	+	-	+	+	-	85-225	225-414	27-62	85-225
7	M	6.4	HLA-id	-	AML I	TBI/Cy +AraC	CsA/MTX	-	-	-	-	-	-	-	-	-	-	-	+	-	27-57	162+	<27	<57
8	M	7.3	HLA-id	-	ALL II	TBI/Cy +VP16	CsA/MTX	-	-	-	-	+	-	-	+	+	-	+	-	-	57-99	302+	57-176	57-176
9	F	7.8	HLA-id	-	ALL II	TBI/Cy +VP16	CsA/MTX	-	-	+	r	-	-	-	+	+	-	+	+	-	27-57	176+	<27	27-57
10	F	6.9	HLA-id	-	ALL II	TBI/Cy +VP16	CsA/MTX	-	-	+	-	+	+	s	+	+	-	-	-	-	27-64	232+	78-232	<64
11	F	4.8	MUD	+[3]	MDS	TBI/Cy +AraC	CsA/MTX	-	-	-	-	-	+	-	+	-	-	-	+	-	152-194	131-194	<26	<131
12	M	1.3	Haplo-id	+	AUL	Bu/Cy	CsA/MTX	-	-	-	-	-	-	-	+	+	-	+	-	-	139-377	377+	377+	377+
13	M	8.3	HLA-id	-	ALL II	TBI/Cy +VP16	CsA/MTX	a l[5]	-	-	-	-	-	-	-	-	-	-	+	-	62-90	202-321	<34	<90

No.	Sex	Age[1]	Donor	TCD	Diagnosis	Conditioning	GvH-prophylaxis	GvHD	CMV[2]				HSV				EBV				VZV				Reconstitution after BMT[4]			
									D	R	Cl		D	R	Cl		D	R	Cl		D	R	Cl		T	Th	Ts/c	B
14	F	1.8	MUD	+	AML II	TBI/Cy +AraC	CsA/MTX	-	+	-	-		-	-	-		+	-	-		-	-	-		174-391	174-391	83-174	83-174
15	M	6.8	HLA-id	-	MDS	TBI/Cy +AraC	CsA/MTX	-	-	+	r		-	+	-		+	+	-		+	+	-		85-188	188-356	<85	85-188
16	F	9.7	MUD	+	MDS	TBI/Cy +AraC	CsA/MTX	-	-	-	-		+	n	s		+	n	-		+	n	-		28-63	364+	<28	<63
17	M	13.1	HLA-id	-	ALL I	TBI/Cy +VP16	CsA/MTX	-	-	-	-		+	-	-		+	n	-		+	+	-		183-463	463+	57-183	183-463
18	M	9.5	HLA-id	-	AML I	TBI/Cy +AraC	CsA/MTX	-	n	-	-		+	+	-		+	+	-		+	+	-		377-610	377+	<85	85-183
19	F	2.1	HLA-id	-	AML I	Bu/Cy +AraC	CsA/MTX	-	-	-	-		-	+	-		-	+	-		-	+	-		27-57	372+	<27	<92
20	F	12	MUD	+	AML I	TBI/Cy +AraC	CsA/MTX	-	+	-	-		+	-	-		+	+	-		-	+	-		27-62	356+	27-62	197-356
21	M	3.3	MUD	-	Ph'CML	TBI/Cy +AraC	CsA/MTX	limchr	-	+	r		n	-	-		n	-	-		n	+	g		94-171	94-171	<34	241+
22	M	11.8	MUD	+	MDS	TBI/Cy +AraC	long course MTX	-	-	+	-		n	-	-		n	+	-		n	+	-		307+	307+	62-111	<48

[1] Age at BMT in years. [2] Viral serology in the donor (D) and the recipient (R) scored as either positive (+), negative (-), or not determined (n), and absence (-) or presence (r = reactivation; s = stomatitis; g = generalized skin eruption) of clinical problems (Cl) related to viral reactivation and/or disease (for definition see Materials and Methods section). [3] T-cell depletion (≥2 log) was achieved by albumin gradient centrifugation and E-rosette sedimentation [18]. Time interval in days after BMT where p_s of age-matched reference values was crossed; < indicates that crossing occurred before this first study sample was taken; + indicates that crossing occurred after this las study sample was taken, so there is a censored observation. T = T-lymphocytes, Th = helper T-lymphcoytes, Ts/c = suppressor/cytotoxic T-lymphocytes as estimated by T-Th, B = B-lymphocytes. [5] a I = acute grade I; limchr = limited chronic.

ALL = acute lymphoid leukemia; AML = acute myeloid leukemia; AraC = cytarabine; AUL = acute undifferentiated leukemia; Bu = busulphan; CMV = cytomegalovirus; CsA = ciclosporin A; Cy = cyclophosphamide; EBV = Epstein-Barr virus; GvH(D) = graft versus host (disease); Haplo-id = haplo-identical; HLA-id = HLA-identical; HSV = herpes simplex virus; MDS = myelodysplastic syndrome; MTX = methotrexate; MUD = matched unrelated donor; Ph'CML = Philadelphia chromosome positive chronic myeloid leukemia; TBI = total body irradiation; TCD = T-cell depletion; VP16 = etoposide; VZV = varicella zoster virus.

TABLE 2. Immunophenotyping protocol.

Marker	FITC-conjugated MoAb (Company[1])	Marker	PE-conjugated MoAb (Company[1])	Recognized lymphocyte subpopulation
IgG1				
CD7	IgG1 (IT)	IgG2	IgG2 (BD)	Isotype control
CD3	Leu-9 (BD)	CD3	Leu-4 (BD)	Total CD3+; CD3+/CD7- T-lymphocytes
CD7	Leu-4 (BD)	CD4	OKT4 (OD)	Total CD3+; CD3+/CD4+ helper T-lymphocytes
CD45RA	Leu-9 (BD)	CD4	OKT4 (OD)	CD4+/CD7- T-lymphocytes
CD45RO	Leu-18 (BD)	CD4	OKT4 (OD)	CD4+/CD45RA+ "naive" helper T-lymphocytes
CD3	UCHL-1 (DK)	CD4	OKT4 (OD)	CD4+/CD45RO+ "memory" helper T-lymphocytes
	Leu-4 (BD)	CD8	OKT8 (OD)	Total CD3+; CD3+/CD8+ suppressor/cytotoxic T-lymphocytes
CD7				
CD20	Leu-9 (BD)	CD8	OKT8 (OD)	CD8+/CD7- lymphocytes
	OKB20 (OD)	CD5	Leu-1 (BD)	Total CD20+; CD20+/CD5+ B-lymphocytes

[1] BD = Becton Dickinson San Jose, CA; DK = Dako, Glostrup, Denmark; IT = Immunotech, Marseille, France; OD = Ortho Diagnostics, Raritan, NJ.

account that only the time interval was known in which the recovery took place, i.e. the data were so-called interval censored.[21] The time-intervals are shown in Table 1: e.g. for patient no. 1, the recovery of helper T-lymphocytes took place somewhere between day 28 and day 77, whereas for patient no. 2 reconstitution had not yet occurred at the last day of follow-up (day 167); this patient is censored. The curves for the cumulative risk on reconstitution were estimated for the total group as well as for patients with and without CMV-reactivation (CMV+ and CMV-), with an HLA-identical (HLA-id) and with a matched unrelated donor (MUD), and with and without T-cell depletion (TCD) (TCD+ and TCD-), separately. The comparison of the groups of CMV+ and CMV- patients, the groups with an HLA-id and a MUD donor, and the groups of TCD+ and TCD- transplants, is expressed as the relative risk (RR) of reaching the p_5 of age-matched reference values, and its 95% confidence interval. The analysis of interval censored data is not available in standard statistical packages. It was done using the GAUSS package and the Turnbull algorithm.[22]

RESULTS

Clinical course (see also Table 1)

Six children developed CMV-reactivation within 2 months posttransplant (5 HLA-id, 1 MUD), none had overt disease. Two children suffered from HSV-stomatitis, and one child developed generalized VZV-related skin eruptions; they were treated successfully with iv aciclovir. There were no EBV-related problems in the study group. Grade I acute graft versus host disease (GvHD) was observed in 1 child (HLA-id), limited chronic GvHD developed in another child (MUD). Due to the limited occurrence of complications in these 22 children, the influence of the development of GvHD or herpesvirus-reactivation on lymphocyte reconstitution after BMT could not be analyzed, except for CMV-reactivation (see below).

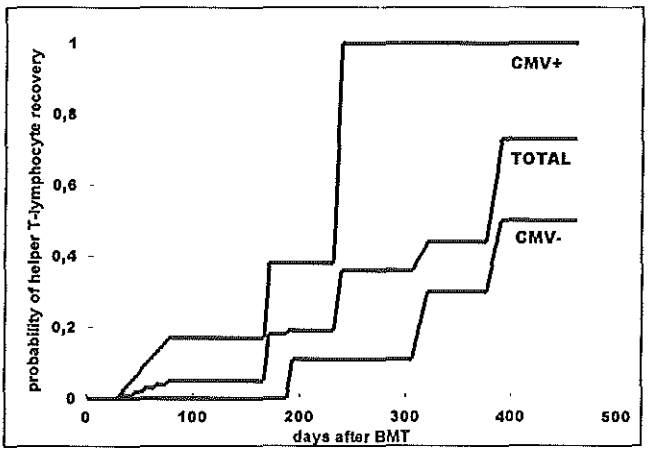

Figure 1. The influence of CMV-reactivation on the probability of CD4+ helper T-lymphocyte recovery (≥ p_5 of age-matched reference values) after BMT in 22 children transplanted for a hematological malignancy.

Recovery of the main lymphocyte populations (see also Table 1)

The absolute lymphocyte counts reached the p_5 of age-matched reference values within 3 months after BMT in 9 patients (41%; 7 HLA-id, 2 MUD/TCD+), and in another 6 patients (27%; 4 HLA-id, 1 MUD/TCD+, 1 MUD/TCD-) between 3-6 months after BMT. The absolute counts of B-lymphocytes reached the p_5 of age-matched reference values within 3 months after BMT in 13 patients (59%; 11 HLA-id, 2 MUD/TCD+), and in another 4 patients (18%; 2 HLA-id, 2 MUD/TCD+) between 3-6 months after BMT. In 5 patients (23%; all HLA-id), absolute counts of B-lymphocytes above the 95th percentile (p_{95}) were reached from 6 months after BMT onwards. The absolute counts of T-lymphocytes reached the p_5 of age-matched reference values within 3 months after BMT in only 3 patients (14%; all HLA-id), and in another 4 patients between 3-6 months after BMT (18%; 2 HLA-id, 2 MUD/TCD+). The absolute counts of CD8+ suppressor/cytotoxic T-lymphocytes (measured in 13 HLA-id and 3 MUD patients only) recovered quickly. They reached the p_5 of age-matched reference values within 3 months after BMT in 10 patients (63%; 8 HLA-id, 1 MUD/TCD+ and 1 MUD/TCD-), and in another 4 patients (25%; 3 HLA-id, 1 MUD/TCD+) between 3-6 months after BMT. CD4+ helper T-lymphocytes remained very low up to 18 months after BMT, despite the fact that all BMT-reciplents were children. They reached the p_5 of age-matched reference values within 3 months after BMT in only 1 patient (5%; HLA-id), and in only 1 more patient (5%; HLA-id) between 3-6 months after BMT. There was no significant difference in the rate of recovery of the main lymphocyte subpopulations between HLA-id and MUD transplants, or between TCD+ and TCD- transplants (Table 3). The 1 haplo-id/TCD+ transplanted patient did not show any recovery of the main lymphocyte subpopulations in the first 6 months after BMT.

The influence of CMV-reactivation

Two patients (nos. 1 and 3; both HLA-id) showed an initial peak in their total

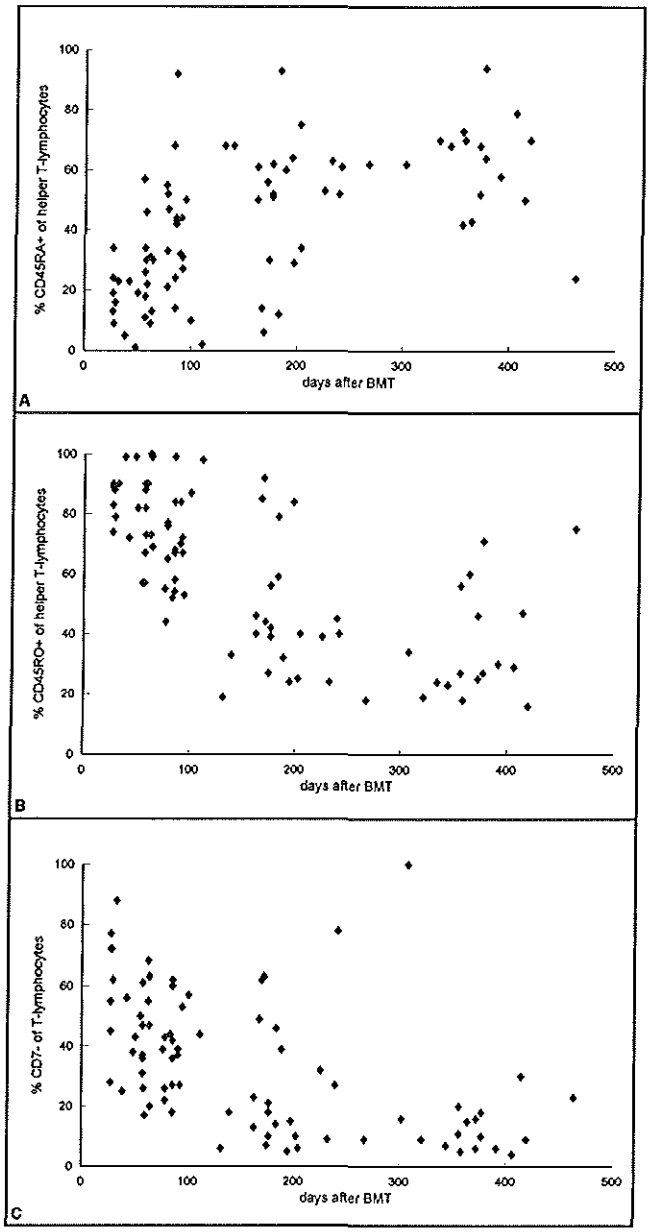

Figure 2. Follow-up of T-lymphocyte subpopulations after BMT in 22 children transplanted for a hematological malignancy. *A*, Percentage CD45RA+ of CD4+ helper T-lymphocytes. *B*, Percentage of CD45RO+ of CD4+ helper T-lymphocytes. *C*, Percentage CD7- of T-lymphocytes.

lymphocyte counts early posttransplant (7.3 x 10^9/l at +42 days and 5.3 x 10^9 /l at +50 days, respectively); both were excreting CMV in their urine, and pp65 was detectable in their blood leukocytes at that time. They also showed an initial peak

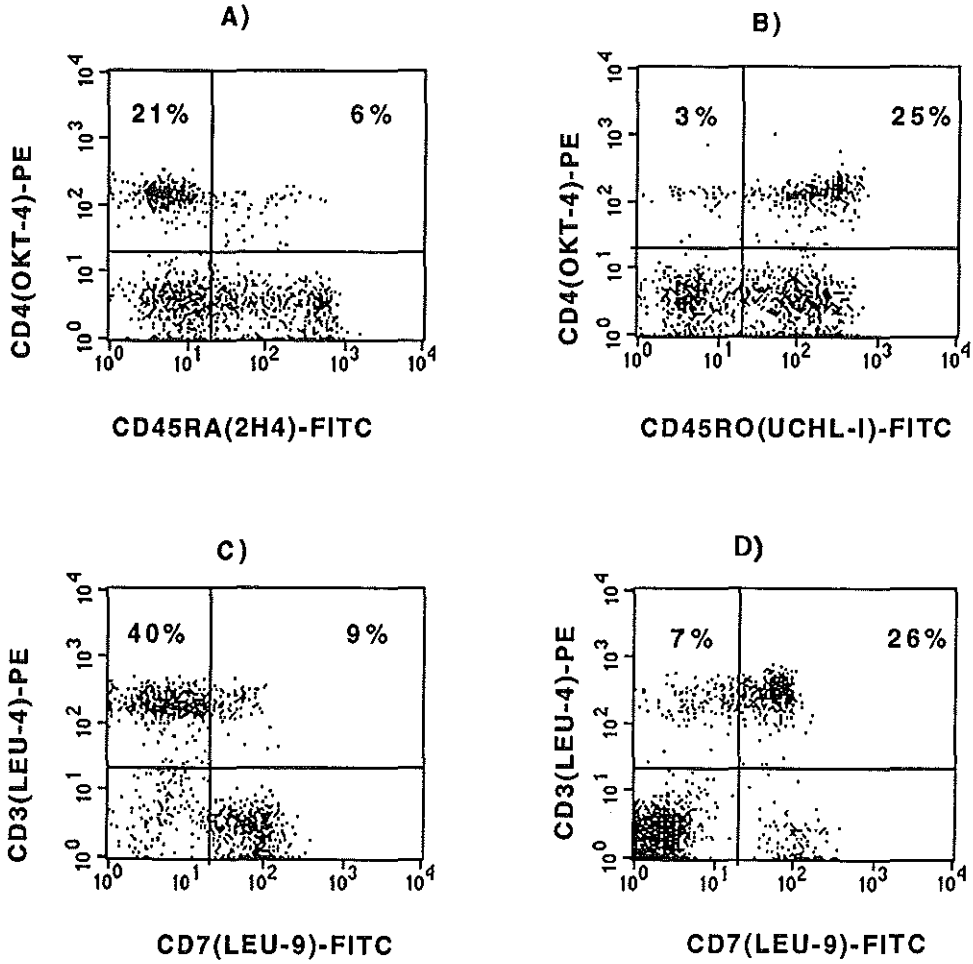

Figure 3. Dot plots of representative peripheral blood samples from the study group of 22 children who were transplanted for a hematological malignancy. *A*, Low percentage CD45RA+ of CD4+ helper T-lymphocytes 2 months after BMT. *B*, High percentage of CD45RO+ of CD4+ helper T-lymphocytes 2 months after BMT. *C*, High percentage of CD7- T-lymphocytes 1 month after BMT. D) Low percentage of CD7- T-lymphocytes 6 months after BMT.

in their T-lymphocyte counts (4.5 resp. 4.4 x 10^9/l), but not in their CD4+ helper T-lymphocyte count. An additional child (no. 21; MUD/TCD-) with CMV-reactivation had very high T-lymphocyte levels at 8 months after BMT (4.2 x 10^9/l). In all these cases the peak reflects an expansion of CD8+ suppressor/cytotoxic T-lymphocytes.

Although there was a trend towards faster quantitative T-lymphocyte recovery among the children with CMV-reactivation, this was not significantly different from

the recovery of T-lymphocytes in children without CMV-reactivation: relative risk CMV+ vs CMV- 2.36, 95% confidence interval 0.82-6.70. Estimated suppressor/cytotoxic T-lymphocyte recovery (assessed as total T-lymphocyte minus CD4+ helper T-lymphocyte counts) was faster among the children with CMV-reactivation: relative risk CMV+ vs CMV- 3.48, 95% confidence interval 1.12-10.6 (Table 3). Unexpectedly, CD4+ helper T-lymphocyte recovery was significantly faster in children with CMV-reactivation: relative risk 12, 95% confidence interval 2.1-90 (Table 3 and Figure 1). To assess whether the faster reconstitution of T-lymphocytes was related to CMV-specific activation, PBMC available from child no. 21 at different time points after BMT were *in vitro* stimulated with CMV. A clear, but low, CMV-specific proliferative response was only observed at +220 days posttransplant. Also, the combined analysis of cell surface expression of CD69 and intracellular expression of IFNγ by flow cytometry indicates only a marginal, if relevant, presence of CMV specific T-lymphocytes at any of these time points. There was no influence of CMV-reactivation on B-lymphocyte recovery (relative risk 1.01, 95% confidence interval 0.31-2.57) (Table 3).

CD45RA and CD45RO expression on helper T-lymphocytes

Early posttransplant, the percentage of helper T-lymphocytes expressing the "naive" CD45RA isoform was low, showing an increase (Figure 2a) from a median of 20% in the first blood samples at 1-2 months (Figure 3a) to a median of 50-60% at >3 months after BMT. BMT donors (see Material and Methods) showed a median percentage of helper T-lymphocytes expressing the "naive" CD45RA isoform of 75%, 66%, and 64% at 1-5 years, 5-10 years, and 10-18 years of age, respectively. The percentage of helper T-lymphocytes expressing the "memory" CD45RO isoform was high, showing a decrease (Figure 2b) from a median of around 90% (TCD- median 84%, TCD+ median 92%) in the first blood samples at 1-2 months (Figure 3b) to a median of 30-40% at >3 months after BMT. BMT donors showed a median percentage of helper T-lymphocytes expressing the "memory" CD45RO isoform of 18%, 32%, and 34% at 1-5 years, 5-10 years, and 10-18 years of age, respectively. This pattern of expression of the CD45 isoforms after BMT is in accordance with data from the literature.[4,28] However, the *absolute counts* of CD45RO+ helper T-lymphocytes did not change significantly during the follow-up period from 1 to 18 months after BMT (median 0.1 x 10^9/l). The absolute counts of CD45RA+ helper T-lymphocytes increased tenfold, but remained low in number, from a median of 0.02 x 10^9/l in the first 6 months after BMT to a median of 0.2 x 10^9/l thereafter.

Predominance of CD7- T-lymphocytes early posttransplant

In the first blood samples at 1 month after BMT, CD7 was not expressed on a median of around 70% of T-lymphocytes (HLA-id/TCD- median 62%, MUD/TCD+ 1 sample 77%) (Figure 3c). The percentage of CD7- cells within the T-lymphocyte population decreased quickly after BMT (Figure 2c) to a median of 40% at 2-3 months, 20% at 6 months (Figure 3d), and 10% in the second year posttransplant. BMT donors showed a median percentage of CD7- cells within the T-lymphocyte population of 5%, 7%, and 7% at 1-5 years, 5-10 years, and 10-18 years of age,

TABLE 3. Influence of CMV-reactivation, donor type and TCD on lymphocyte recovery.

Lymphocyte subpopulation		Day after BMT at which the cumulative incidence of crossing the p_5 -value of age-matched reference values reaches 20%, 50%, and 80%, respectively.			Relative Risk of crossing the p_5 -value of age-matched reference values of CMV+ versus CMV- children, HLA-id versus MUD transplants, and TCD+ versus TCD- grafts, respectively.	
		20%	50%	80%	Relative Risk	95% Confidence interval
T-lymphocytes	Total[1]	28	90	187		
	CMV-[2]	47	90	380		
	CMV+[3]	28	28	141	2.36	0.82-6.70
	HLA-id[4]	28	87	185		
	MUD[5]	45	157	383	0.66	0.17-1.58
	TCD-[6]	28	88	99		
	TCD+[7]	45	186	334	0.58	0.16-1.54
Helper T-lymphocytes	Total	233	380	594		
	CMV-	313	391	780		
	CMV+	168	234	237	12	2.1-90
	HLA-id	235	382	765		
	MUD	25	381	387	1.32	0.28-5.17
	TCD-	233	239	732		
	TCD+	194	383	388	0.58	0.10-2.44
Suppressor/cytotoxic T-lymphocytes[8]	Total	11	28	97		
	CMV-	12	28	102		
	CMV+	11	26	28	3.48	1.12-10.6
	HLA-id	9	23	94		
	MUD	16	28	95	1.10	0.41-2.57
	TCD-	11	28	88		
	TCD+	9	21	109	0.40	0.10-1.12
B-lymphocytes	Total	36	42	90		
	CMV-	39	42	90		
	CMV+	36	42	90	1.01	0.31-2.57
	HLA-id	36	41	89		
	MUD	37	87	286	0.73	0.24-1.84
	TCD-	36	41	89		
	TCD+	37	87	330	0.59	0.17-1.59

[1] All children after allogeneic BMT.[2] Children without CMV-reactivation (n=16).[3] Children with CMV-reactivation (n=6).
[4] Children transplanted with an HLA-id donor (n=15). [5]Children transplanted with a MUD donor (n=6).
[6]Children transplanted with a full bone marrow graft (n=16).
[7]Children transplanted with a T-cell depleted bone marrow graft (n=6).
[8] Assessed as total T-lymphocyte minus CD4+ helper T-lymphocyte counts.
Abbreviations: BMT = bone marrow transplantation; CMV = cytomegalovirus; HLA-id = HLA-identical; MUD = matched unrelated donor; TCD = T-cell depletion.

TABLE 4. CMV-specific *in vitro* stimulation of PBMC of the CMV+ patient no. 21.

		Stimulus CMV (AD169) infected HEL-cells	Stimulus: uninfected HEL-cells
Proliferation			
PBMC day	+80	0.3[1]	0.1
	+220	3.2	0.2
	+264	0.1	0.2
Percentage of CD3+ T-lymphocytes expressing IFNγ and CD69			
PBMC day	+80	0.011[2]	0.002
	+220	0.002	0.003
	+264	0.002	0.006

[1] counts per minute x 10^{-3} of ^{3}H-thymidine incorporation after 6 days of culture
[2] Percentage of CD3+ T-lymphocytes expressing intracellular IFNγ and cell surface CD69 after 6 hours of stimulation
Abbreviations: CMV = cytomegalovirus; IFN = interferon; PBMC = peripheral blood mononuclear cells.

respectively. At 1 month after BMT the median absolute count of CD7-/CD3+ T-lymphocytes was 0.5 x 10^{9}/l, thereafter it did not change significantly during the remaining follow-up period from 2 to 18 months after BMT (median 0.1- 0.2 x 10^{9}/l). The median absolute count of CD7-/CD3+ T-lymphocytes in BMT donors was 0.1 x 10^{9}/l in all age groups. Six patients showed high absolute counts of CD7-/CD3+ T-lymphocytes in the first 3 months after BMT (1.0-2.5 x 10^{9}/l). Four of these patients suffered from CMV-reactivation. The median absolute count of CD7+/CD3+ T-lymphocytes slowly increased from 0.4 x 10^{9}/l at 1 month after BMT to 1.6 x 10^{9}/l at 12-18 months after BMT. CD7 expression on CD4+ helper T-lymphocytes and on CD8+ cells followed a similar pattern (data not shown).

CD5+ B-lymphocytes

Although the median percentage of CD5+ cells within the B-lymphocyte population decreased from 65% (median) at 1-2 months after BMT (HLA-id median 65%, MUD/TCD+ 2 samples 46% and 48%) to 42% (median) at ≥ 1 year after BMT, the absolute counts of CD5+/CD20+ B-lymphocytes remained stable during the entire follow-up period (median 0.3 x 10^{9}/l at 1-2 months after BMT to 0.4 x 10^{9}/l at ≥ 1 year after BMT).

DISCUSSION

We prospectively analyzed the reconstitution of lymphocyte subpopulations after allogeneic BMT in a group of 22 children transplanted for a hematological malignancy. The recovery, measured in terms of crossing the p_5 of age-matched reference values,[17] was slow. In one third of the children, the absolute lymphocyte counts still had not reached the p_5 of age-matched reference values 6 months after BMT. In addition, B- and T-lymphocytes showed a different pattern of recovery.

The absolute counts of B-lymphocytes recovered relatively fast after BMT,

reaching the p_5 within 6 months posttransplant in 77% of the children. A quarter of the children, all transplanted with an HLA-id donor, reached values above the p_{95}, resembling normal ontogeny during the first year of life, as was described before.[8] CD5+ B-lymphocytes, which are presumed to be more primitive cells,[23] seem to repopulate the periphery faster than CD5- "conventional" B-lymphocytes following BMT: although the median percentage of CD5+ cells within the B-lymphocyte population was high early after BMT, as was described before,[24] the *absolute counts* of CD5+/CD20+ B-lymphocytes remained stable during the entire follow-up period. CD5- B-lymphocytes increased only slowly, resembling normal ontogeny in the first year of life (E. de Vries et al., submitted).

The absolute counts of T-lymphocytes recovered more slowly: in only one third of the children levels above the p_5 were reached at 6 months after BMT. At that time, only 2 children (9%; both HLA-id) had reached levels above the p_5 for CD4+ helper T-lymphocytes, whereas 88% of the children had reached levels above the p_5 for CD8+ suppressor/cytotoxic T-lymphocytes. This very slow reconstitution of CD4+ helper T-lymphocytes was described before in adults, [5] but not in children.[15] However, Foot et al [15] did not apply age-matched reference values to evaluate the reconstitution of lymphocyte subpopulations in their study. The p_5 of the absolute counts of CD4+ helper T-lymphocytes is higher in younger children, resulting in an increasing threshold for "recovery", with decreasing age. The comparatively slow recovery of CD4+ helper T-lymphocytes after BMT in children and adults is in contrast with the conclusions of Mackall et al.[13] Their findings led to the conclusion that the thymus-dependent reconstitution of helper T-lymphocytes *after cytostatic therapy* is delayed in adults in comparison with children. Maybe, the pre-BMT conditioning regimen initially damages the thymus to such an extent that CD4+ helper T-lymphocyte development is impaired for a long period of time in pediatric bone marrow graft recipients as well. CD8+ suppressor/cytotoxic T-lymphocytes rapidly recovered to normal absolute counts after allogeneic BMT in the children, despite the potential damage to the thymus of the myelo-ablative therapy, but this was also described in adult BMT recipients[9] and after intensive chemotherapy.[13,14]

Unexpectedly, we found a clear positive influence of CMV-reactivation on quantitative CD4+ helper T-lymphocyte recovery. CMV represents a significant problem in the posttransplant period, with a high morbidity and mortality, especially in adults.[25] In healthy individuals, primary infection results in lymphocytosis of CD8+ cytotoxic T-lymphocytes (CTL's) during a mild or even subclinical illness.[26] These CTL's are probably responsible for elimination of the active infection, and induction of protective immunity. A lymphocytosis of CD8+ cytotoxic T-lymphocytes was described in children after TCD+ BMT.[16] We also found a faster recovery of suppressor/cytotoxic T-lymphocytes, assessed as total T-lymphocyte minus CD4+ helper T-lymphocyte counts, in children with CMV-reactivation. The faster CD4+ helper T-lymphocyte recovery we found in children with CMV-reactivation might be related to the more benign course of CMV-reactivation in children than in adults: Reusser et al[27] found a significant association between the presence of CMV-specific T-helper cell proliferation and the development of CTL activity. We did not find a significant CMV-specific proliferation of T-lymphocytes

after BMT in the one child with CMV-reactivation we tested, however.

Our data confirm the findings by others[4,28] of a high percentage of "memory" CD45RO+ helper T-lymphocytes and a low percentage of "naive" CD45RA+ helper T-lymphocytes early posttransplant. However, our data also show that a different picture emerges when *absolute counts* are considered. Actually, the "memory" CD45RO+ helper T-lymphocyte counts remain stable during a follow up period from 1 to 18 months after BMT, and the "naive" CD45RA+ helper T-lymphocyte counts increase slowly. Interestingly, the few T-lymphocytes that were present at 1 month after BMT were also largely CD7-, as was recently described in adults.[29] In normal individuals, a small subpopulation of CD7-, mostly CD4+, CD45RO+ and CD45RA- primed "memory" T-lymphocytes exists, which are probably destined for homing to the skin. This subpopulation increases in size with age.[30] The absolute counts of CD7+ T-lymphocytes increased only slowly after BMT, whereas the absolute counts of CD7- T-lymphocytes remained stable during 18 months of follow-up.

In conclusion, we found that immune reconstitution after BMT, especially of CD4+ helper T-lymphocytes, is a slow process in children, as it is in adults. Unexpectedly, CD4+ helper T-lymphocyte recovery was significantly enhanced in children following CMV-reactivation. We did not find evidence of a peripheral expansion (i.e. increase of blood lymphocyte subpopulation *absolute counts*) of previously primed donor-derived CD45RO+ and CD7- "memory" T-lymphocytes from 1 month after BMT onward in these children. Of course, it is possible that those lymphocytes that appear around 1 month after BMT are the result of such a peripheral expansion during the first month posttransplant. It is difficult to analyse this with immunophenotyping, due to the extremely low numbers of lymphocytes in the first month after BMT. Like in adults, there was but a slow reconstitution of truly "naive" CD45RA+ T-lymphocytes coming out of the thymus.

REFERENCES

1 Vossen JMJJ. Bone Marrow Transplantation in children. *Baillière's Clin Pediatr* 1994;1:1029-1059.
2 Sanders JE. Bone marrow transplantation for pediatric malignancies. *Pediatr Clin North Am* 1997;44:1005-1020.
3 Serody JS, Shea TC. Prevention of infections in bone marrow transplant recipients. *Inf Dis Clin North Am* 1997;11:459-477.
4 Storek J, Witherspoon RP, Storb R. T cell reconstitution after bone marrow transplantation into adult patients does not resemble T cell development in early life. *Bone Marrow Transplant* 1995;16:413-425.
5 Lum LG. The kinetics of immune reconstitution after human marrow transplantation. *Blood* 1987;69:369-380.
6 Atkinson K. Reconstitution of haematopoietic and immune systems after marrow transplantation. *Bone Marrow Transplant* 1990;5:209-226.
7 Leino L, Lilius EM, Nikoskelainen J, et al. The reappearance of 10 differentiation antigens on peripheral blood lymphocytes after allogeneic bone marrow transplantation. *Bone Marrow Transplant* 1991;8:339-344.
8 Storek J, Ferrara S, Ku N, *et al.* B cell reconstitution after human bone marrow transplantation: recapitulation of ontogeny? *Bone Marrow Transplant* 1993;12:387-398.
9 Verma UN, Mazumder A. Immune reconstitution following bone marrow transplantation. *Cancer*

Immunol Immunother 1993;37:351-360.
10 Storek J, Gooley T, Witherspoon RP, et al. Infectious morbidity in long-term survivors of allogeneic marrow transplantation is associated with low CD4 T cell counts. *Am J Hematol* 1997;54:131-138.
11 Heitger A, Neu N, Kern H, et al. Essential role of the thymus to reconstitute naive (CD45RA+) T-helper cells after human allogeneic BMT. *Blood* 1997;90:850-857.
12 Pilarski LM, Gillitzer R, Zola H, et al. Definition of the thymic generative lineage by selective expression of high molecular weight isoforms of CD45 (T200). *Eur J Immunol* 1991;19:589-597.
13 Mackall CL, Fleisher TA, Brown MR, et al. Age, thymopoiesis, and CD4+ T-lymphocyte regeneration after intensive chemotherapy. *N Engl J Med* 1995;332:143-149.
14 Mackall CL, Fleisher TA, Brown MR, et al. Distinctions between CD8+ and CD4+ T-cell regenerative pathways result in prolonged T-cell subset imbalance after intensive chemotherapy. *Blood* 1997;89:3700-3707.
15 Foot ABM, Potter MN, Donaldson C, et al. Immune reconstitution after BMT in children. *Bone Marrow Transplant* 1993;11:7-13.
16 Kook H, Goldman F, Padley D, et al. Reconstitution of the immune system after unrelated or partially matched T cell-depleted bone marrow transplantation in children: immunophenotyic analysis and factors affecting the speed of recovery. *Blood* 1996;88:1089-1097.
17 Comans-Bitter WM, de Groot R, van den Beemd R, et al. Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. *J Pediatr* 1997;130:388-393.
18 Wagemaker G, Heidt PJ, Merchav S, Bekkum DW van. Abrogation of histo-incompatibility barriers in Rhesus monkeys. In: Baum SJ, Ledney GD, Thierfelder S (eds.). *Experimental Haematology Today.* Karger, Basel, 1982, pp 11-118.
19 Hebart H, Muller C, Loffler J, et al. Monitoring of CMV infection: a comparison of PCR from whole blood, plasma-PCR, pp65-antigenemia and virus culture in patients after bone marrow transplantation. *Bone Marrow Transplant* 1996;17:861-868.
20 Waldrup et al. *J Immunol* 1996.
21 Klein JP, Moeschberger ML. *Survival analysis. Techniques for censored and truncated data.* Springer, New York, 1997.
22 Turnbull BW. Nonparametric estimation of a survivorship function with double censored data. *JASA* 1974;69:169-173.
23 Herzenberg LA, Kantor AB, Herzenberg LA. Layered evolution in the immune system. A model for the ontogeny and develoment of multiple lymphocyte lineages. In: CD5 B cells in development and disease. Proceedings of a conference. *Ann NY Acad Sci* 1991;651:1-9.
24 Small TN, Keever CA, Weiner-Fedus S, et al. B-cell differentiation following autologous, conventional, or T-cell depleted bone marrow transplantation: a recapitulation of normal B-cell ontogeny. *Blood* 1990;76:1647-1656.
25 Winston DJ. Infections in bone marrow transplant recipients. In: Mandell GL, Bennett JE, Dolin R (eds). *Principles and practice of infectious diseases.* Churchill Livingstone, New York, 1995, pp 2717-2722.
26 Locksley RM, Wilson CB. Cell-mediated immunity and its role in host defense. In: Mandell GL, Bennett JE, Dolin R (eds). *Principles and practice of infectious diseases.* Churchill Livingstone, New York, 1995, pp 102-149.
27 Reusser P, Riddell SR, Meyers JD, Greenberg PD. Cytotoxic T-lymphocyte response to cytomegalovirus after human allogeneic bone marrow transplantation: pattern of recovery and correlation with cytomegalovirus infection and disease. *Blood* 1991;78:1373-1380.
28 Gorla R, Airo P, Ferremi-Leali P, et al. Predominance of 'memory' phenotype within CD4+ and CD8+ lymphocyte subsets after allogeneic BMT. *Bone Marrow Transplant* 1993;11:346-347.
29 Leblond V, Ben Othman T, Blanc C, et al. Expansion of CD4+CD7- T cells, a memory subset with preferential interleukin-4 production, after bone marrow transplantation. *Transplantation* 1997;64:1453-1459.
30 Reinhold U, Abken H. CD4+ CD7- T cells: A separate subpopulation of memory T cells? *J Clin Immunol* 1997;17:265-271.

CHAPTER 10

IDENTIFICATION OF AN UNUSUAL FcγRECEPTOR IIIa (CD16) ON NATURAL KILLER CELLS IN A PATIENT WITH RECURRENT INFECTIONS

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Blood 1996;88:3022-3027

ABSTRACT

We found an unusual Fcγ Receptor IIIa (CD16a) phenotype on the natural killer (NK) cells of a 3-year-old boy, who suffered from recurrent viral respiratory tract infections since birth. He also had severe clinical problems after Bacille Calmette-Guérin (BCG) vaccination and following Epstein-Barr virus and varicella zoster virus infections. His peripheral blood lymphocytes contained a normal percentage and absolute number of CD3-CD7+ cells, which were positively stained with the CD16 monoclonal antibodies (MoAbs) 3G8 and CLBFcRgran1, but did marginally stain with the CD16 MoAb Leu11c/B73.1. FcγRIIIb expression on granulocytes appeared to be normal. NK-cell function, analyzed *in vitro* by direct cytotoxicity on K562 target cells and ADCC-activity on P815 target cells, was normal compared with an age-matched healthy control. Sequence analysis of the FcγRIIIA gene, encoding CD16 on NK-cells and macrophages, showed a T to A nucleotide substitution at position 230 on both alleles, predicting a leucine (L) to histidine (H) amino acid change at position 48 in the first extracellular Ig-like domain of FcγRIIIa, which contains the Leu11c/B73.1 epitope. The combined use of CD16 and CD56 MoAbs labeled with the same fluorescent dye, as often applied in routine immunophenotyping procedures, will leave these homozygotes undiagnosed. The pattern of infections in this patient is in agreement with the postulated function of NK-cells in the immunological defense against viruses and other intracellular microorganisms. Further analysis of the NK-cell function *in vitro*, and follow-up of the clinical course of FcγRIIIA-48H/H homozygotes is required to ascertain whether this genotype is causally related to an NK-cell immunodeficiency.

INTRODUCTION

Natural killer (NK) cells are generally defined as large granular lymphocytes that express CD16 and/or CD56, and are negative for pan-T- and B-cell markers. They are able to lyse sensitized target cells *in vitro* through the mechanism of antibody-dependent cell-mediated cytotoxicity (ADCC) and to kill K562 target cells spontaneously. Their function in vivo is not fully known. NK-cells probably play a role in the destruction of tumor cells and in the resistance to infections caused by certain viruses and other intracellular pathogens.[1]

The Fcγ receptor IIIa (CD16) is involved in the triggering of ADCC[1,2] and spontaneous cytotoxicity.[3,4] Recently, an expression polymorphism of CD16 on NK-cells derived from healthy individuals, based on a difference in the number of binding sites for monomeric IgG per NK-cell, has been described.[5] Until recently, no genetic polymorphism of FcγRIIIa had been reported.[6]

Here, we describe a 3-year-old boy who suffered from recurrent viral respiratory tract infections since birth. He also had severe problems with Bacille Calmette-Guérin (BCG) vaccination, and Epstein-Barr virus and varicella zoster virus infections. This clinical pattern might be compatible with an in vivo dysfunction of NK-cells. Immunophenotyping of peripheral blood mononuclear cells (PBMC)

indicated that NK-cells, defined as CD3-CD7+ lymphocytes, are normally present, but that the CD16 molecule expressed by these cells lacks the epitope recognized by the MoAb Leu11c/B73.1. The *in vitro* NK-cell function was normal compared with an age-matched healthy control. Sequence analysis of the FcγRIIIA gene revealed a T to A nucleotide substitution at position 230 on both alleles, predicting a leucine to histidine change at amino acid position 48 in the first extracellular domain of FcγRIIIa.

MATERIALS AND METHODS

Patient

The patient was born at term after an uneventful pregnancy and delivery. He was the first-born child of non-consanguineous parents of Turkish and Dutch-Norwegian descent. Recently, a second son was born in this family. From 3 months of age onward, the patient suffered from recurrent, mainly viral, upper respiratory tract infections. These were accompanied by wheezing and nocturnal dyspnea, which reacted well to inhaled salbutamol and, later on, to inhaled steroids. BCG vaccination at 6 months of age resulted in local abscess formation, fever, and malaise, only cured 1 year later by excision of the abscess and 2 months of isoniazid therapy. At 18 months of age, he developed a prolonged Epstein-Barr virus infection with fever and malaise, which lasted about 10 months. He mounted a normal antibody response to Epstein-Barr virus during this period. At 30 months of age, he had chickenpox, which was progressive during 2 weeks, and was finally cured with acyclovir therapy. Despite all this, his growth and development were normal.

The absolute number and function of his peripheral blood granulocytes, T-, and B-cells were normal, as were the levels of serum Ig isotypes and specific antibodies following diphteria toxoid and tetanus toxoid-inactivated polio virus type I, II, and III (DT-IPV) vaccination. However, the CD3-CD7+ NK-cells were not stained by the CD16 MoAb Leu11c/B73.1. NK-cell function was tested by spontaneous cytotoxicity of K562 target cells and by ADCC of P815 targets, and further investigation of the FcγRIIIa (CD16) expression on the NK-cells and of the FcγRIIIA gene was undertaken.

Isolation of cells

Fresh anticoagulated blood was diluted 1:2 in phosphate-buffered saline (PBS) and centrifuged over a Percoll gradient with a specific gravity of 1.076 g/ml (Pharmacia, Uppsala, Sweden). Mononuclear cells (PBMC) were harvested from the interphase and the pellet, containing mainly neutrophils and erythrocytes, was treated with ice-cold NH_4Cl solution (155 mmol/L NH_4Cl, 10 mmol/L $KHCO_3$, 0.1 mmol/L EDTA, pH 7.4) to lyse the erythrocytes.

MoAbs

The following MoAbs were used: pan-FcγRIII CD16 MoAbs: CLBFcRgran1 (mIgG2a), 3G8 (mIgG1), Leu11a (mIgG1), DJ130c (mIgG1), BW209/2 (mIgG2a);

NA1-FcγRIIIb plus FcγRIIIa CD16 MoAbs: B73.1 (mIgG1), phycoerythrin (PE)-labeled Leu11c (mIgG1); NA2-FcγRIIIb plus FcγRIIIa CD16 MoAb: GRM1 (mIgG2a); less-glycosylated NA2-FcγRIIIb plus FcγRIIIa CD16 MoAb: PEN1 (mIgG2a). The MoAbs Leu11c/B73.1, GRM1, PEN1 and BW209/2 recognize epitopes located at the first extracellular Ig-like domain of CD16, whereas the epitopes recognized by the MoAbs CLBFcRgran1, 3G8 and Leu11a reside at the membrane-proximal Ig-like domain. BW209/2 was a gift from Dr R. Kurrle, Behring Werke, Marburg, Germany. B73.1 was kindly provided by Dr B. Perussia, Thomas Jefferson University, Philadelphia, PA. PE-labeled Leu11c (clone B73.1) was obtained from Becton Dickinson, San Jose, CA. The other CD16 MoAbs were obtained via the Vth Leukocyte Typing Workshop. PE- and fluorescein isothiocyanate (FITC)-labeled Leu4 (CD3; mIgG1) and PE-labeled Leu19 (CD56; mIgG1) were obtained from Becton Dickinson. Biotin-labeled CD7 (mIgG2a), FITC-labeled goat-anti-mouse-Ig, and murine control MoAbs of the IgG1 and IgG2a subclass with irrelevant specificities (mIgG1 and mIgG2a, respectively) were from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands.

Flowcytometry

PBMC and neutrophils were incubated with CD16 MoAbs for 25 minutes at room temperature. After washing with PBS containing 0.2% bovine serum albumin (BSA) (wt/vol), the cells were incubated with FITC-labeled goat antimouse Ig for 25 minutes at room temperature. In triple color experiments, the free $F(ab)_2$ parts of FITC-labeled goat antimouse Ig were first blocked with a mixture of control mIgG1 and mIgG2a. Thereafter, PE-labeled Leu4 (CD3) and biotin-labeled CD7 MoAbs were added. Binding of CD7-biotin was detected with Cy-Chrome-labeled streptavidin (Pharmingen, San Diego, CA). Immunofluorescent staining was assessed by flow cytometry (FACScan, Becton Dickinson). A gate was set around the lymphocyte population on the basis of forward and orthogonal light scatter characteristics and staining patterns with the combination of CD45 and CD14 M-oAbs.[7]

Cytotoxicity assays

Cytolytic activities of PBMC were determined in standard 3-hour ^{51}Cr-release assays.[8] Briefly, varying numbers of lymphocytes were seeded in triplicate in 96-well, round-bottomed microtiter plates (150 µL/well). A fixed number of target cells labeled with ^{51}Cr (200 µCi/10^6 cells) was added in a volume of 100 µL/well. At the end of the incubation period (37 °C and 5% CO_2), release of ^{51}Cr into the supernatants was measured. The following target cells were used: for the assessment of NK activity, the K562 erythromyeloid leukemia line, and for ADCC, the P815 mouse mastocytoma cell line coated with rabbit anti-P815 IgG. The cytotoxxicity assays were performed at four different effector to target (E:T) ratios, ie, 50:1, 25:1, 12.5:1 , and 6.25:1. The percentage of specific lysis (SL) was calculated according to the formula:

$$\%SL = \frac{\text{Exp.cpm - Spon.cpm} \times 100}{\text{Max.cpm - Spon.cpm}}$$

where Exp. Is the experimental number of counts obtained from target cells

incubated with effector cells; Spn. Is the spontaneously released counts obtained from targets incubated in medium alone; and Max. is the maximal counts obtained from targets lysed with a 1% Triton X-100 solution.

Sequence analysis

Messenger RNA was isolated from mononuclear cells and reversely transcribed into cDNA. Primers used to amplify the entire coding region[9] of FcγRIIIa-encoding cDNA were: Tf1 (sense; nt -5-21) 5'-CGC AAG CTT TGG TGA CTT GTC CAC TC-3' and Tf2 (antisense; nt 963-988) 5'-CGC TCT AGA TCA TGG GCT TTT CCC TT-3'. The polymerase chain reaction (PCR) amplified fragment was ligated into a pGEM-T vector, according to the manufacturer's instructions (Promega, Madison, WI). After cloning into *E. coli*, the insert was amplifiedand the nucleotide sequence was determined by cycle sequencing with ^{32}P end-labeled primers (Amersham, Amersham, UK), using the BRL cycle sequencing kit according to the manufacturer's instructions (BRL, Gaithersburg, MD). The following primers were used to sequence the entire coding region[9]: sense direction: Tf1 (see above), NA-L (nt 106-125), P306 (nt 322-342) and Sn (nt 658-679); anti-sense direction: Tf2 (see above), P664 (nt 658-679) and NA-R (nt 329-348).

FcγRIIIA gene-specific fragment amplification and FcγRIIIA genotyping

Amplification of an FcγRIIIA gene-specific fragment containing the site under investigation was achieved by means of an allele-specific primer annealing (ASPA) assay. The sense primer IIIA1 (5'-CAC AGT GGT TTC ACA ATG AGA G-3') was compatible with NA2-FcγRIIIB and FcγRIIIA. The antisense primer IIIA2 (5'-CTG TAC TCT CCA CTG TGG TC-3') annealed completely to NA1-FcγRIIIB and FcγRIIIA. The PCR assay was performed with 1 µg of genomic DNA, 150 ng of each primer, 200 µmol/L of each dNTP and 2 U of Taq-DNA polymerase (Promega), diluted in a buffer recommended by the manufacturer in a total volume of 50 µL in a Perkin-Elmer Cetus Cycler (Norwalk, CT). The first cycle consisted of 5 minutes of denaturation at 95 °C, 1 minute of primer annealing at 64° C and 1 minute of extension at 72 °C. This was followed by 35 of these cycles in which the denaturation time was decreased to 1 minute. The final cycle was followed by 9 minutes at 72 °C to complete extension.

The 91-bp product was electrophoresed in 10% polyacrylamide gels, stained with ethidium bromide and visualized with ultraviolet (UV) light. Cycle sequencing of the amplified fragments was performed with primer IIIA2 as described above.

RESULTS

Flowcytometry

The PBMC of the patient contained 9% CD3-CD7+ lymphocytes and 7% CD3-CD56+ lymphocytes, respectively, indicating that NK-cells are present in normal relative numbers (Figure 1). As shown in Figure 2, the CD3-CD7+ lymphocytes

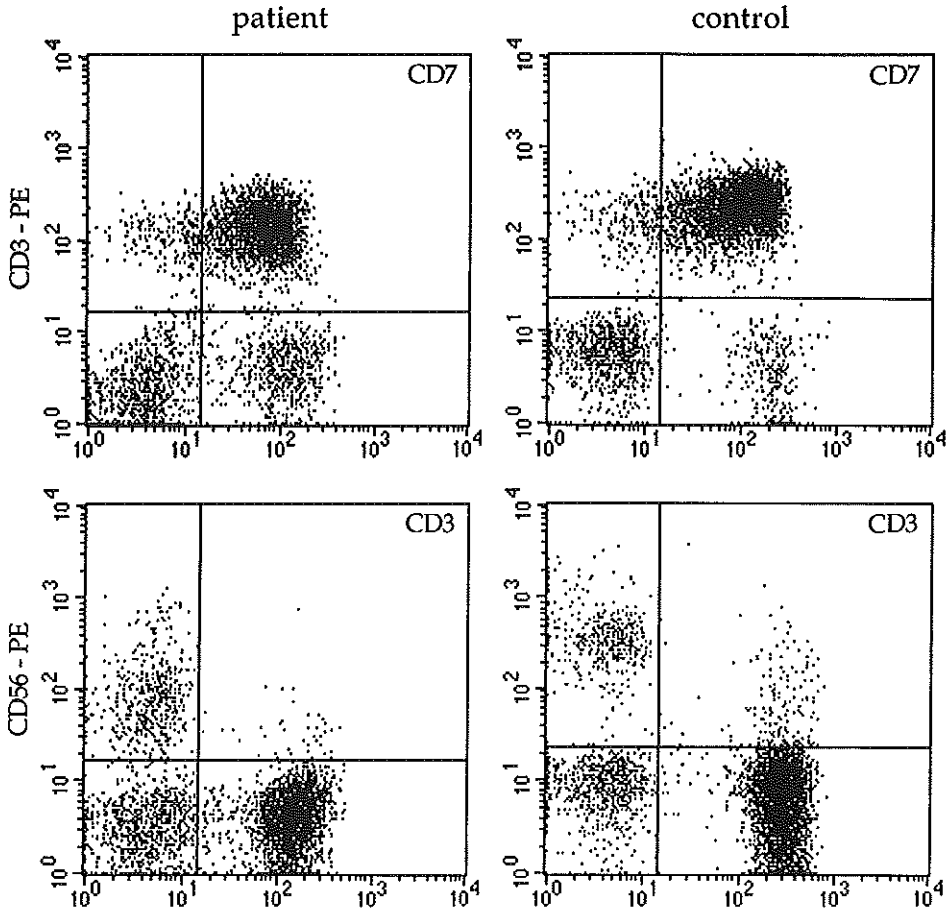

Figure 1. Staining pattern of lymphocytes with CD3, CD7, and CD56 MoAbs. The lymphocyte gated mononuclear cells of the patient contained 9% CD3-CD7+ lymphocytes and 7% CD3-CD56+ lymphocytes, indicating that NK-cells are present in a relative number, which is comparable to that of the healthy, unrelated, age-matched control.

stained minimally with the CD16 MoAb B73.1. Therefore, we tested several other CD16 MoAbs. CD3-CD7+ lymphocytes were positively stained with CLBFcRgran1, indicating that FcγRIIIa was expressed on the cell membrane. The results of the complete flow cytometric analysis of CD3-CD7+ lymphocytes of the patient, his family members, and a healthy, unrelated, control are summarized in Table 1. Except for Leu11c/B73.1, the CD3-CD7+ lymphocytes of the patient and his newborn brother stained positively with the applied CD16 MoAbs. NK-cells of both parents stained positively with B73.1, albeit with a somewhat lower fluorescence intensity compared with the control. Flow cytometric analysis of the patient's granulocytes showed a normal reactivity pattern with CD16 MoAbs and an NA(1+2+) phenotype, which was confirmed by genotyping. Granulocytes of his mother and brother were also NA(1+2+), his father had an NA(1-2+) phenotype.

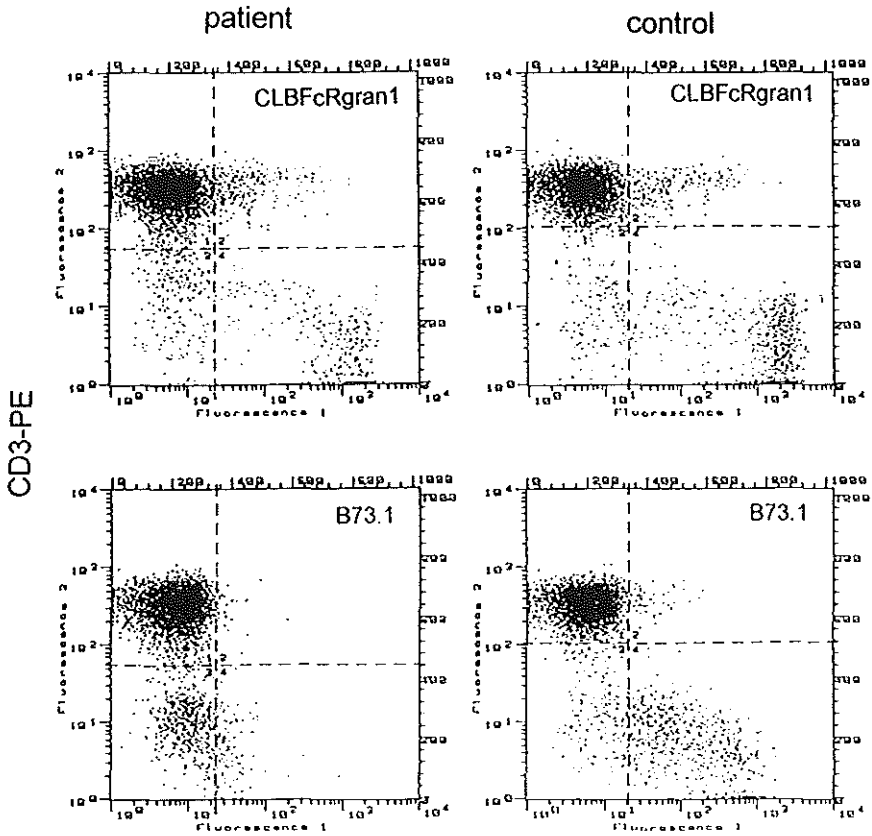

Figure 2. Staining pattern of lymphocytes with CD16 MoAbs. Staining pattern of lymphocytes of the patient and a healthy, unrelated, control with CD16 MoAbs. With CD16 MoAb B73.1 followed by FITC-labeled goat antimouse Ig, staining of the CD3- (CD7+) subpopulation is severely decreased in comparison with the control. However, there is a clearly positive subpopulation when the CD16 MoAb CLBFcRgran1 is used.

Cytotoxicity assays

Spontaneous cytotoxicity of K562 target cells and ADCC of P815 targets measured by a ^{51}Cr release assay were normal for the patient and his younger brother, as compared with an age-matched healthy control (Figure 3).

Sequence analysis

Sequencing of the entire coding region of FcγRIIIa-encoding cDNA of the patient revealed a T to A nucleotide substitution at position 230 on both alleles. This substitution predicts a leucine to histidine change at amino acid position 48 in the first extracellular domain of FcγRIIIa. As shown in Figure 4, cycle sequencing of the FcγRIIIA-specific PCR products of the parents revealed that they both were heterozygous for this nucleotide substitution (FcγRIIIA-48L/H). Like the patient, the second son also had an FcγRIIIA-48H/H genotype (data not shown).

TABLE 1. Immunofluorescent staining of CD3-CD7+ cells with CD16 MoAbs[1]

	FcγRIIIA genotype									
	48-H/H (patient)		*48-L/H* (father)		*48-L/H* (mother)		*48-H/H* (brother)		*48-L/L* (control)	
CD16MoAab	%pos	MFI	%pos	MFI	%pos	MFI	%pos	MFI	%pos	MFI
B73.1	9	136	71	251	63	251	14	181	71	387
CLBFcRgran1	62	1,372	92	1,703	72	1,643	48	1,276	78	1,899
3G8	65	889	91	991	72	889	46	889	71	991
Leu-11a	55	432	87	374	66	1,027	43	416	77	556
DJ130c	54	402	75	360	65	432	39	335	67	234
BW209/2	63	858	90	1,231	74	1,231	37	827	79	1,474
GRM1	61	889	88	1,372	67	1,276	49	1,104	76	1,529
PEN1	57	691	88	956	56	991	40	827	74	1,104

[1] Percentage of CD3-/CD7+ cells stained positively with the indicated CD16 MoAbs and mean intensity of fluorescence (channel values, MFI: 4 log decade, relative linear units) of the patient, his family, and a healthy, unrelated, control. Staining of CD3-/CD7+ cells with MoAbs of irrelevant specificities was less than 1.0% for the mIgG1 and less than 4.0% for the mIgG2a control MoAbs.

DISCUSSION

Only one patient with an absolute NK-cell deficiency has been described until now. This was an adolescent with recurrent life-threatening herpes-virus infections, who completely lacked CD16 and/or CD56 positive cells in vivo and NK-activity *in vitro*.[10] Although NK-cell function is impaired in patients with Chediak-Higashi syndrome and Leucocyte Adhesion Deficiency disease, this impairment does not dominate their clinical course.[1]

Here, we describe a patient with an as yet unknown FcγRIIIA-48H/H genotype, caused by a T to A nucleotide substitution at position 230 on both alleles. This particular genotype leads to an unusual phenotype, with loss of the Leu11c/B73.1 epitope of the Fcγ Receptor IIIa (CD16) on NK-cells (and, presumably, on macrophages). At nucleotide position 230 of the FcγRIIIA gene a biallelic polymorphism (T or G) has recently been found, and further analysis, prompted by the findings in this patient, revealed that, in fact, a triallelic polymorphism (T, G or A) exists at this position, with gene frequencies in Caucasians of 86, 6, and 8%, respectively.[6] With this frequency of the 230A allele, it can be predicted that 6.4 FcγRIIIA-48H/H homozygotes per thousand individuals should be present in the Caucasian population. It is important to realize that this Leu11c/B73.1 negative phenotype will not be detected in most routine immunophenotyping procedures. NK-cells are mostly defined as the CD16 and/or CD56 positive and CD3 negative lymphocyte population. A CD16 (most frequently Leu11c) and a CD56 MoAb labeled with the same fluorescent dye are then used simultaneously. The lack of reactivity with Leu11c/B73.1 will remain unnoticed, as a large part of the NK-cell population is CD56 positive, as well. This can explain why this phenotype was not described before.

The question of whether the unusual Fcγ Receptor IIIa and the clinical

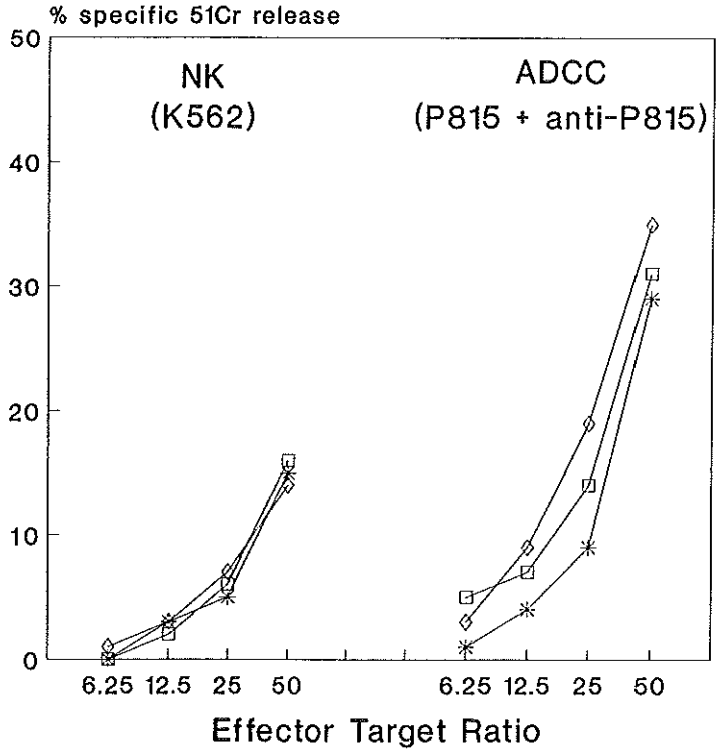

Figure 3. NK and ADCC cytolytic activities of lymphocytes. Spontaneous cytotoxicity of K562 target cells and ADCC of P815 targets were measured in a ^{51}Cr release assay using different effector:target ratios. There was no difference between the patient (□), his brother (◊), and a healthy, unrelated, age-matched control (*).

problems in this patient are causally related cannot be answered on the basis of the available data. Although the infectious problems of the patient described here fit into the postulated role of NK-cells in in vivo immune surveillance, the NK-cell activity as measured *in vitro* was not affected. In addition, the binding site of FcγRIIIa for IgG was described to be located at the second, membrane-proximal, extracellular Ig-like domain, whereas the Leu11c/B73.1 epitope is located at the first extracellular Ig-like domain of FcγRIIIa, albeit not at amino acid position 48.[11] The epitope recognized by 3G8 and CLBFcRgran1 is located at the second extracellular Ig-like domain. On the other hand, it has also been described that B73.1 inhibits IgG binding to FcγRIIIa.[12]

The FcγRIIIA-48H/H genotype with loss of the Leu11c/B73.1 epitope of the FcγRIIIa (CD16) on NK-cells was also present in the younger brother of the patient. He suffers from recurrent, mainly viral, upper respiratory tract infections as well. Also, he has food allergy and eczema. He has not yet come into contact with either Epstein-Barr or varicella zoster virus. Very recently, a 2-year old boy of Caucasian descent, not related to the patient described here, with the same geno- and phenotype was identified. He was born prematurely as one of a triplet and had suffered from food allergy and eczema, like the younger brother of the patient described here. This boy was tested after an unusually severe and prolonged infection that looked like chickenpox. Varicella zoster virus was not isolated, and

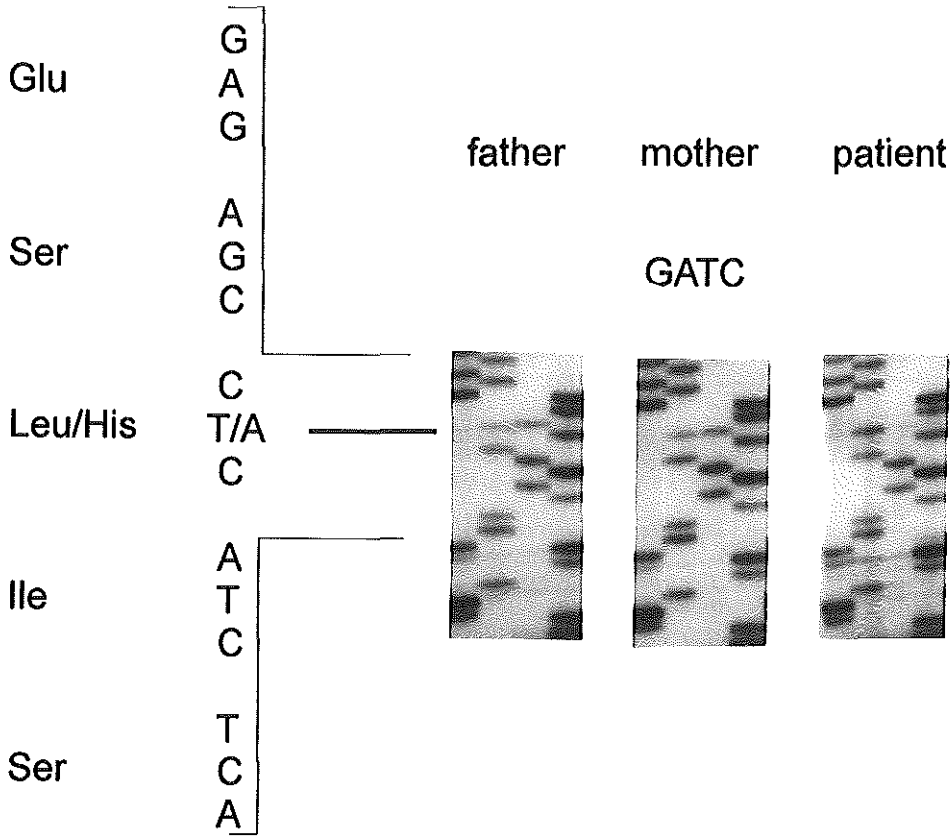

Figure 4. Sequence analysis of the FcγRIIIA gene. A T to A nucleotide substitution at position 230 in the FcγRIIIA gene predicts a leucine to histidine amino acid change at position 48 of the first extracellular Ig-like domain of CD16, which contains the Leu11c/B73.1 epitope. The parents are heterozygous, and the patient is homozygous for this nucleotide substitution.

he did not mount an antibody response to this virus.

The identification of this unrelated boy with the same unusual Fcγ Receptor IIIa and problems with combating an infection could make a causal relationship between the FcγRIIIA genotype and the clincial presentation described here more likely. Bearing in mind the relatively high allelic frequency of 8% in the Caucasian population, it could be that other intrinsic factors, besides FcγRIIIA-48H/H homozygosity, need to be present before these infectious problems occur. More elaborate analysis of the NK-cell function *in vitro*, and follow-up of the clinical course of newly traced FcγRIIIA-48H/H homozygotes in the population is required to ascertain whether this genotype is really the cause of an NK-cell immunodeficiency.

NOTE ADDED IN PROOF

While our report was being reviewed, another patient with FcγRIIIA-48H/H homozygosity was published by Jawahar et al.[13]

REFERENCES

1 Robertson MJ, Ritz J. Biology and clinical relevance of human natural killer cells. *Blood* 1990;76:2421-2438.

2 Van de Winkel JGJ, Anderson CL. Biology of human immunoglobulin G Fc receptors. *J Leukocyte Biol* 1991;49:511-524.

3 Gherman M, Manciulea M, Bancu AC, Sulica A, Stanworth DR, Herberman RB. Regulation of human natural cytotoxicity by IgG. I. Characterization of the structural site on monomeric IgG responsible for inhibiting natural killer cell activity. *Mol Immunol* 1987;24:743-750.

4 Bonnema JD, Karnitz LM, Schoon RA, Abraham RT, Leibson PJ. Fc Receptor stimulation of phosphatidylinositol 3-kinase in natural killer cells is associated with protein kinase C-independent granule release and cell-mediated cytotoxicity. *J Exp Med* 1994;180:1427-1435.

5 Vance BA, Huizinga TWJ, Wardwell K, Guyre PM. Binding of monomeric human IgG defines an expression polymorphism of FcγRIII on large granular lymphocyte/natural killer cells. *J Immunol* 1993;151:6429-6439.

6 de Haas M, Koene HR, Kleijer M, de Vries E, Simsek S, van Tol MJD, Roos D, von dem Borne AEGKr. A triallelic Fcγ receptor type IIIA polymorphism influences the binding of human IgG by NK cell FcγRIIIa. *J Immunol* 1996;156:2948-2955.

7 Loken MR, Brosnan JM, Bach BA, Ault KA. Establishing optimal lymphocyte gates for immunophenotyping by flow cytometry. *Cytometry* 1990;11:453-459.

8 Bolhuis RLH, Van de Griend RJ. PHA induced proliferation and cytolytic activity in T3+ but not in T3- cloned T lymphocytes, requires the involvement of the T3 antigen for signal transmission. *Cell Immunol* 1985;93:46-57.

9 Ravetch JV, Perussia B. Alternative membrane forms of FcγRIII (CD16) on human natural killer cells and neutrophils. Cell type-specific expression of two genes that differ in single nucleotide substitutions. *J Exp Med* 1989;170:481-497.

10 Biron CA, Byron KS, Sullivan JL. Severe herpesvirus infections in an adolescent without natural killer cells. *N Engl J Med* 1989;320:1731-1735.

11 Hibbs ML, Tolvanen M, Carpén O. Membrane-proximal Ig-like domain of FcγRIII (CD16) contains residues critical for ligand binding. *J Immunol* 1994;152:4466-4474.

12 Perussia B, Trinchieri G. Antibody 3G8, specific for the human neutrophil Fc receptor, reacts with natural killer cells. *J Immunol* 1984;132:1410-1415.

13 Jawahar S, Moody C, Chan M, Finberg R, Geha R, Chatila T. Natural killer (NK) cell deficiency associated with an epitope-deficient Fc receptor IIIA (CD16-II). *Clin Exp Immunol* 1996;103:408-413.

CHAPTER 11

TCRαβ+ T-LYMPHOCYTOPENIA IN TWO SIBLINGS

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ABSTRACT

Two siblings, a boy and a girl, suffered from recurrent respiratory tract infections and episodes of intractable diarrhea associated with weight loss. The boy also suffered from severe varicella zoster virus pneumonia and Epstein-Barr virus related B-cell lymphoma. Immunological studies revealed lymphopenia and decreased proliferative response upon stimulation of lymphocytes *in vitro*. TCRαβ+ T-lymphocyte counts were low compared to age-matched controls, with CD4+/CD45RA+ "naive" helper T-lymphocyte counts being most severely affected. We believe that these two siblings do not fit into any of the clinical syndromes reported to date, and that they might suffer from an as yet undescribed type of immunodeficiency disease.

INTRODUCTION

Knowledge of the clinical presentation, immunological characteristics and genetic defects of a large variety of primary immunodeficiency diseases has rapidly expanded in the past decade,[1] and new clinical entities continue to be described.[e.g.2]

Here, we present two immunodeficient siblings showing TCRαβ+ T-lymphocytopenia with CD4+/CD45RA+ "naive" helper T-lymphocytes being most severely affected. We believe that these children do not fit into any of the immunological syndromes reported to date.

PATIENTS

A boy, now aged 11 years, and his sister, now aged 8 years, were born to healthy non-consanguineous Caucasian parents. Both children were vaccinated without complications according to the regular Belgian schedule, including live-attenuated oral poliovirus vaccination. Since early childhood, they suffered from recurrent bacterial otitis media and bronchitis. At 6 months of age, the girl was admitted with bacterial sepsis and skin abscesses.

Both children showed difficulties in dealing with herpes viruses. At 4 years of age, the boy developed varicella zoster virus pneumonia and required mechanical ventilation as well as i.v. aciclovir. When he was 5 years of age, a high-grade B-cell lymphoma of the lung was diagnosed after he seroconverted to Epstein-Barr virus (EBV). This tumor was probably EBV-related, because EBV-mRNA (EBER) was detected in the tumor cells. The boy was treated successfully with cytostatic therapy according to the EORTC-CLCG 58881 ALL protocol after surgical resection of the tumor. At 3 years of age, the girl was evaluated for persistent generalized lymphadenopathy, but no malignancy was found. In the girl, cytomegalovirus could still be cultured from the throat and urine several years after seroconversion. IgM-VCA was still present in her serum several years after

TABLE 1. Immunological investigations.

Immunophenotyping		*Boy (9 yrs)*	*Girl (6 yrs)*	*Normal range*[1]
Total lymphocytes		0.7[2]	0.9	1.1-5.9
CD3+ T-lymphocytes		0.4	0.8	0.7-4.2
CD3+/CD4+ Th-subset[3]		0.2	0.5	0.3-2.0
CD3+/CD8+ Ts/c-subset[3]		0.1	0.2	0.3-1.8
CD19+/CD20+ B-lymphocytes		0.2	0.2	0.2-1.6
CD3-/CD16+ and/or CD56+ NK-cells		0.1	0.2	0.09-0.9
T-lymphocytes:	%TCRγδ+[4]	17	29	
	%CD25+	9	1	
CD4+ Th-subset:	%CD45RA+	4	3	
	%CD45RO+	99	98	
	%HLA-DR+	22	12	
	%CD27+	34	49	
CD8+ Ts/c-subset:	%CD45RA+	53	49	
	%CD45RO+	76	71	
	%HLA-DR+	28	18	
	%CD27+	29	9	

In vitro proliferation		*Boy (9 yrs)*	*% of controls*	*Girl (3.5 yrs)*	*% of controls*	*Girl (4 yrs)*	*% of controls*
PHA		5.6[6]	22	15.4	61	0.7	5
PWM						3.4	21
ConA						2.5	39
Allo-Ag						2.0	9
CD3						4.2	22
CD3 + rIL2						28.0	29
Coated CD3	alone	9.7	36	10.2	38	12.0	14
	+CD28 McAb					38.1	37
	+rIL2	16.6	61	19.7	73	38.7	41
Coated CD2	alone					0.8	1
	+CD28 McAb					33.1	32
	+rIL2					24.6	31
PMA	alone					0.9	7
	+CD28 McAb					18.4	47
	+rIL2					10.8	45
	+Ca-ionophore	5.6	143	8.2	210		

[1] Age-matched reference values [3].
[2] Absolute counts (x 10^9/l).
[3] Th-subset = helper T-lymphocytes; Ts/c-subset = suprressor/cytotoxic T-lymphocytes.
[4] TCRγδ is the alternative T-cell receptor, CD25 (IL2 receptor α-chain) and HLA-DR (class II) are markers of activation, CD45RA is expressed on "naive" T-lymphocytes, CD45RO is expressed on "memory" T-lymphocytes, CD2 es expressed on mature and activated T-lymphocytes.
[5] [3] H-thymidine incorporation as a percentage of the mean response of two healthy adult controls investigated simultaneously.
[6] ^{3}H-thymidine incorporation (x 10^3) after stimulation of 40,000 peripheral blood mononuclear cells (mean of triplicate cultures).

seroconversion to EBV.

Around 4 years of age, episodes of recurrent diarrhea of unknown origin with signs of malabsorption started to occur in both. Their were no signs of autoimmunity. The children are small for age (p_3 for weight and height). Their mental development and school performance are normal.

Interestingly, the father suffers from recurrent mycotic dermatitis (*T. rubrum*) on a sharply demarcated area of the thigh. The mother has no infectious problems.

IMMUNOLOGICAL INVESTIGATIONS

Immunophenotyping of lymphocyte subpopulations (Table 1) showed TCRαβ+ T-lymphocytopenia with CD4+/CD45RA+ "naive" helper T-lymphocytes being most severely affected (Figure 1a). CD8+ suppressor/cytotoxic T-lymphocyte counts were also decreased. No CD4+/CD8+ "double-positive" T-cells or CD1+ T-cells were present in the circulation. β_2 micoglobulin (HLA class I) and HLA-DR (HLA class II) were expressed normally.

Despite the high relative frequency of TCRγδ+ T-lymphocytes, the absolute count of TCRγδ+ T-lymphocytes was normal. PCR/heteroduplex analysis of $V_\gamma 1/V_\gamma 2$-$J_\gamma 1$ and $V_\delta 1/V_\delta 2$-$J_\delta 1$ rearrangements as well as flow cytometric analysis of the pattern of V_γ and V_δ usage as compared to two age-matched controls, indicated the polyclonal composition of this cell population in both.[3,4]

The proliferative responses of peripheral blood mononuclear cells (Table 1) were low. They were similarly affected in the girl and the boy. Although the responses could be upregulated by the addition of recombinant interleukin-2 (rIL2) or CD28 monoclonal antibody (McAb), they remained low in comparison with controls. Normal proliferation was induced through bypassing of the CD3-TCR complex with PMA and Ca-ionophore.

Serum immunoglobulin (Ig) levels were normal in the boy, but serum IgG decreased progressively in the girl (3.6 g/l at 6 years of age) with concomitant development of IgG1, IgG2 and IgG4 subclass deficiency. Serum Igs showed a restricted heterogeneity in the girl and a dominant homogeneous component (M-component) in the boy (Figure 1b). Both children showed a partial response to intramuscular booster immunization with diphteria, tetanus toxoid, and inactivated poliovirus and to Pneumovax immunisation (boy to tetanus, *S. pneumoniae* type 3 and 9, and poliovirus, girl to *S. pneumoniae* type 3 and poliovirus).

Chromosomal analysis showed a normal karyotype, and DNA-analysis gave no indications for the presence of deletions at chromosome 22q11 using the DGCR-N25 probe. Adenosine deaminase and purine nucleotide phosphorylase enzyme levels in red blood cells were also normal.

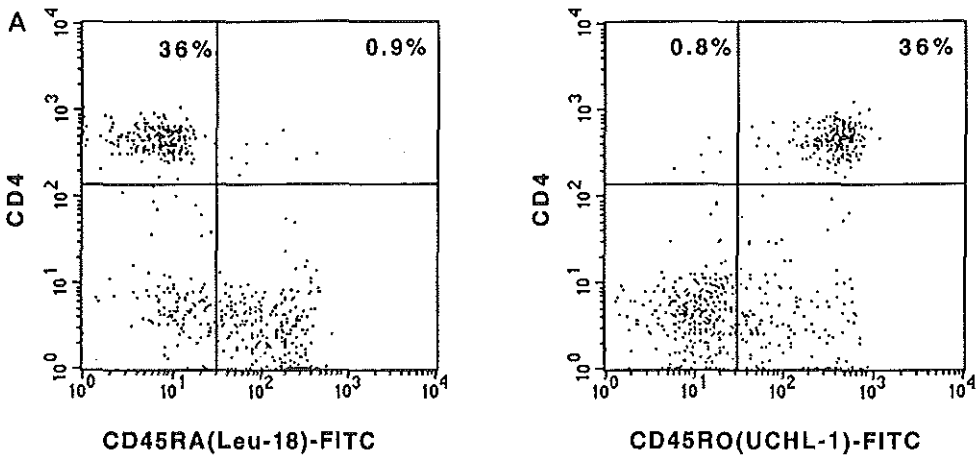

Figure 1. Representative examples of the results of the immunological investigations. *A*, Decreased CD4+/CD45RA+ "naive" helper T-lymphocytes. *B*, Serum immunoglobulin electrophoresis: standardized normal human serum (NHS; upper lane), a dominant homogeneous component in the boy (middle lane), and restricted heterogeneity in the girl (lower lane).

The parents showed no quantitative abnormalities of IgM, total IgG, IgA, and IgG subclasses. TCRαβ+ T-lymphocytes were at the lower limit of normal in the father, and normal in the mother (0.7 x 10^9/l and 1.7 x 10^9/l, respectively).

DISCUSSION

The two siblings show a combined immunodeficiency disease (CID) with possible autosomal recessive inheritance and a relatively mild clinical presentation. Their clinical course suggests a defect of cell-mediated immunity with fulminant varicella zoster virus pneumonia and EBV-related B-cell lymphoma in the boy, and episodes of intractable diarrhea with associated weight loss in both. This does not easily fit into the phenotype of common variable immunodeficiency.[1] The recurrent respiratory tract infections suggest an additional defect in their antibody production, probably secondary to impaired CD4+ helper T-lymphocyte function.

This clinical picture is supported by the results of the immunological laboratory studies. Lymphopenia and decreased *in vitro* proliferation of lymphocytes were found in both children, confirming a defect in cell-mediated immunity. This was accompanied by limited specific antibody responses (both children), a homogeneous IgG component (boy), restricted serum Ig heterogeneity (girl), and IgG deficiency (girl), supporting a disturbance in the control of B-lymphocyte activity by helper T-lymphocytes.

The lymphopenia was mainly due to the low T-lymphocyte count - caused by TCRαβ+ T-lymphocytopenia - with B-lymphocyte and NK-cell counts at the lower limit of normal. Initially, the high relative frequency of TCRγδ+ T-lymphocytes suggested an aberrant TCRγδ+ T-lymphocytosis. This seemed to be confirmed because flow cytometric analysis of V_γ and V_δ usage differed from adult controls. However, the absolute count and the V_γ and V_δ usage of TCRγδ+ T-lymphocytes were normal when compared to age-matched controls. This clearly illustrates the importance of adhering strictly to the use of absolute counts as well as age-matched reference values[5,6] in the analysis of lymphocyte subpopulations in immunodeficient patients.

We can only speculate on the nature of the underlying molecular defect in these siblings. Although CD8+ suppressor/cytotoxic T-lymphocyte counts were also decreased, CD4+/CD45RA+ "naive" helper T-lymphocyte counts were most severely affected. According to recent models of T-cell development,[7] either a defect in the nurturing capacity of the thymic micro-environment leading to a decreased output of "naive" cells, a "leaky" arrest in T-cell development, or premature death in the periphery could be the cause of the lymphopenia in these siblings.

DNA-analysis showed no evidence of DiGeorge anomaly, the only thymic defect described in man, nor did the children suffer from any of the problems generally associated with DiGeorge anomaly (cardiac malformation, hypoparathyroidism). Mutations affecting the thymic stromal environment have been described in mice (winged-helix nude, RelB, Hoxa-3).[7] An as yet undescribed defect in the thymus or an equivalent of one of the mouse mutations is a possibility for the molecular defect in these siblings.

A "leaky" developmental arrest caused by a defect situated in the T-lymphocytes themselves is a distinct possibility. This defect would most likely be situated in the CD3 -TCR complex or associated phosphorylating enzymes, because lymphocyte proliferation could be upregulated substantially by rIL2 or CD28 McAb stimulation, and stimulation with PMA/ionomycin elicited normal proliferation *in vitro*. Although the latter suggests ZAP70 deficiency, the immunophenotyping results and the clinical course in these siblings do not fit in with that diagnosis. In ZAP70 deficiency, a severe clinical course is associated with a selective absence of CD8+ T-lymphocytes and abundant CD4+ T-lymphocytes in the peripheral blood.[8]

It has been described in mice that maintenance of a pool of "resting" T-lymphocytes with a normal life-span requires permanent low-grade activation by continuing subtle stimulation of their TCR molecules by MHC. Mutations which disrupt these subtle signals (LKLF, Ets-1) cause a decrease in the numbers of "resting" T-lymphocytes in mice.[7] Perhaps absence of such continuing subtle stimulation leads to apoptosis. Apoptotic depletion of CD4+ T-cells has been described in patients with idiopathic CD4+ T-lymphocytopenia.[9] The molecular defect in the here described siblings could be a human equivalent to one of the mouse mutations.

REFERENCES

1 Rosen FS, Wedgwood RJP, Eibl M, Fischer A, Aiuti F, Notarangelo L, Kishimoto T, Resnick IB, Hammarstrom L, Seger R, Chapel H, Thompson RA, Cooper MD, Geha RS, Good RA, Waldmann TA. Primary immunodeficiency diseases. Report of a WHO scientific group. *Clin Exp Immunol* 1997;109(S1):1-28.

2 Freier S, Kerem E, Dranitzky Z, Schlesinger M, Rabinowitz R, Brautbar C, Ashkirat M, Naparstek Y. Hereditary CD4+ T lymphocytopenia. *Arch Dis Child* 1998;78:371-372.

3 Langerak AW, Szczepański, Van der Burg M, Wolvers-Tettero ILM, Van Dongen JJM. Heteroduplex PCR analysis of rearranged T cell receptor genes for clonality assessment in suspect T cell proliferations. *Leukemia* 1997;11:2192-2199.

4 Langerak AW, Wolvers-Tettero ILM, Van den Beemd MWM, Van Wering ER, Ludwig W-D, Hählen K, Necker A, Van Dongen JJM. Immunophenotypic and immunogenotypic characteristics of TCRγδ+ T cell acute lymphoblastic leukemia. *Leukemia* 1999;13:206-214.

5 Comans-Bitter WM, De Groot R, Van den Beemd R, Neijens HJ, Hop WCJ, Groeneveld K, Hooijkaas H, Van Dongen JJM. Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. *J Pediatr* 1997;130:388-393.

6 Bonilla FA, Oettgen HC. Normal ranges for lymphocyte subsets in children. *J Pediatr* 1997;130:347-349.

7 Fischer A, Malissen B. Natural and engineered disorders of lymphocyte development. *Science* 1998;280:237-243.

8 Elder ME, Weiss A. SCID resulting from mutations in the gene encoding the protein tyrosine kinase ZAP-70. In: Ochs HD, Smith C.I.E., Puck J.M. (eds). *Primary Immunodeficiency diseases. A molecular and genetic approach.* New York, Oxford University Press, 1999: pp.146-154.

9 Laurence J, Mitra D, Steiner M, Lynch DH, Siegel FP, Staiano-Coico L. Apoptotic depletion of CD4+ T cells in idiopathic CD4+ T lymphocytopenia. *J Clin Invest* 1996;97:672-680.

CHAPTER 12

THE 782C→T (T254M) XHIM MUTATION: LACK OF A TIGHT PHENOTYPE-GENOTYPE RELATIONSHIP

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Submitted

INTRODUCTION

Recently, Seyama et al[1] described twenty-eight unique mutations of the *CD40 ligand (CD40L)* gene in forty-five X-linked hyper IgM syndrome (XHIM) patients from thirty unrelated families. Generally, peripheral blood mononuclear cells (PBMC) are screened for CD40L expression after activation with PMA and ionomycin to diagnose XHIM. Activated PBMC of most XHIM patients did not express functional CD40L (CD154): they failed to bind a CD40-Ig fusion protein (bCD40-Ig). The authors discerned five different CD40L staining patterns on cultured T-cells, using a polyclonal antiserum (pAb), four different monoclonal antibodies (McAb), and bCD40-Ig. The type 1 pattern showed weak staining of CD40L with all reagents mentioned, the type 2-5 patterns successively showed loss of functional activity (bCD40-Ig binding) and protein epitope expression (loss of 1-4 McAb and finally pAb binding). A relationship between genotype and phenotype was suggested, with "milder genotypes" (resulting in staining pattern type 1 or 2) showing milder clinical phenotypes: in five out of ten patients from nine families with staining pattern type 1 or 2, symptoms started relatively late, and none of them suffered from opportunistic infections. Two patients with the 782C→T mutation, a missense mutation in exon 5, fitted into this group.

We found the 782C→T XHIM mutation in four patients from two different families. Although they showed a favourable clinical course after immunoglobulin replacement therapy was started, the two index patients had symptoms from an early age onwards, including one who presented with Pneumocystis carinii pneumonia (PCP). The other two children were diagnosed shortly after birth because of the XHIM index patient in the family.

PATIENTS

The first index patient, patient No.1 (of family A) is a Dutch young man, now aged 19 years, who was treated with immunoglobulin replacement therapy from the age of two years onward because of hypogammaglobulinemia and frequent respiratory tract infections. Since then, the frequency of infections normalized. He never had an infection with an opportunistic pathogen, and never received prophylaxis with Co-trimoxazole. Recently, stimulation of his PBMC with PMA and ionomycin induced expression of the early activation marker CD69 (Leu-23, Becton Dickinson, San Jose, CA) on his CD3+ T-lymphocytes, whereas CD40L (LL48, Schering-Plough, Dardilly, France) expression was absent (Figure 1). Fluorescent sequencing of the *CD40L* gene revealed a 782C→T mutation (expected protein alteration T254M). The family was screened, and in patient No.2, his cousin now aged 2 years, XHIM was identified shortly after birth (Figure 2). At nine months of age, the serum IgG level had decreased to <0.5 g/l. Although there were no clinical symptoms, immunoglobulin replacement therapy was started because of the increased risk of serious infections.

The second index patient, patient No.3 (of family B) is a British boy, now aged

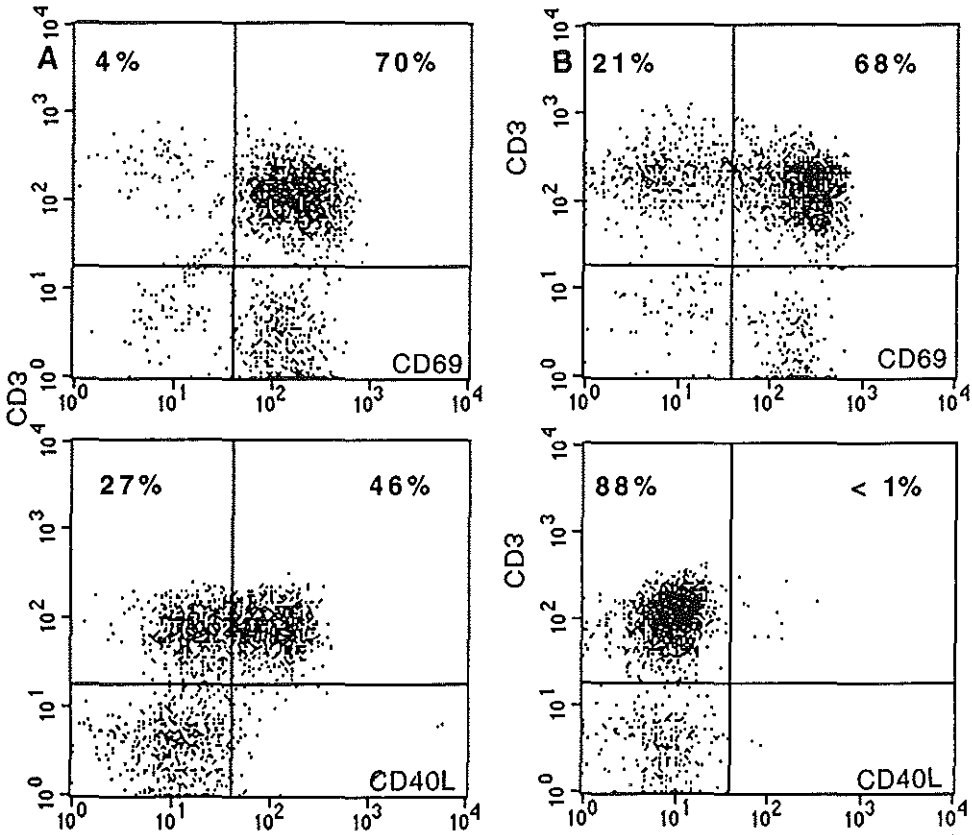

Figure 1. CD69 and CD40L expression on CD3+ T-lymphocytes after stimulation of PBMC with PMA and ionomycin. *A*, Normal control. *B*, Patient No.1.

11 years, who was diagnosed as having XHIM at 6 months of age when he presented with PCP. Following successful treatment of this infection, he has remained asymptomatic on immunoglobulin replacement therapy and Co-trimoxazole prophylaxis except for one episode of a diarrhoeal illness at the age of seven years, which was of unknown etiology, lasted for two months and was associated with weight loss. Recent studies show no evidence of cholangiopathy on liver function tests, liver histology and endoscopic retrograde cholangio-pancreatogram. His younger brother (patient No.4), now aged eight years, was diagnosed soon after birth based on the family history. He has remained completely well on immunoglobulin replacement therapy and Co-trimoxazole prophylaxis. His liver is also normal. The mutation in these boys has been previously reported (case 13 in Katz et al).[2]

DISCUSSION

In our opinion, the issue of a phenotype-genotype relation in XHIM remains

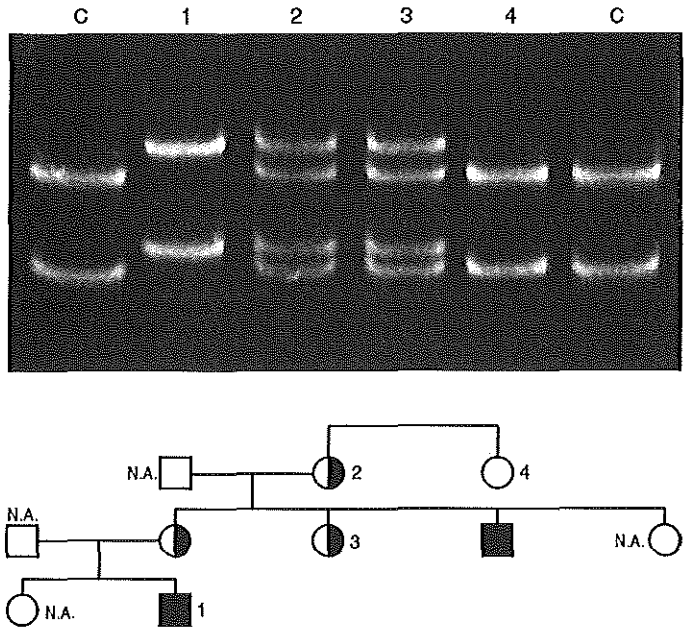

Figure 2. Single strand conformation polymorphism (SSCP) analysis of XHIM family A with T254M mutation. PCR products (198 bp) of exon 5 of the *CD40L* gene were denatured and run on a 12.5% polyacrylamide gel at 150 Volts for 14 hours. The two control (C) lanes show the positions of the DNA strands of the unaffected X-chromosome. The C→T mutation leads to a shift in the position of these bands as is shown in lane 1 (XHIM patient No.2). Carriers (lanes 2 and 3) show both mutated and unmutated DNA strands. Non-carriers (lane 4) only show the unmutated DNA strands.

debatable. Not all patients with a staining pattern type 1 or 2 described by Seyama et al[1] showed a mild clinical phenotype. Comparison of our two index patients with the 782C→T mutation with the two mild patients described by Seyama et al[1] does not support a tight phenotype-genotype relation in XHIM patients, although our four patients all showed a favourable course after immunoglobulin replacement therapy. We agree that symptomatic PCP may occur in non immunocompromised infants, though this is very unusual. Nevertheless, we do not believe that the life threatening episode of PCP in our patient No. 3 is compatible with a label of "mild phenotype".

Furthermore, our XHIM cases also show that a favourable clinical course on immunoglobulin replacement therapy is compatible with a diagnosis of XHIM. This implies that testing of CD40L expression on activated PBMC should be considered in all patients with unexplained hypogammaglobulinemia.

REFERENCES

1 Seyama K, Nonoyama S, Gangsaas I, Hollenbaugh D, Pabst HF, Aruffo A, Ochs HD. Mutations of the *CD40 ligand* gene and its effect on CD40 ligand expression in patients with X-linked hyper IgM syndrome. *Blood* 1998;92:2421-2434.

2 Katz F, Hinshelwood S, Rutland P, Jones A, Kinnon C, Morgan G: Mutation analysis in CD40 ligand deficiency leading to X-linked hypogammaglobulinemia with hyper IgM syndrome. *Hum Mutat* 1996;8:223-228.

CHAPTER 13

A GIRL WITH AGAMMAGLOBULINEMIA AND ABSENT BLOOD B-LYMPHOCYTES

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INTRODUCTION

Agammaglobulinemia with markedly reduced numbers of B-lymphocytes - but normal T-lymphocytes - in the peripheral blood is the characteristic phenotype of X-linked agammaglobulinemia (XLA) which is caused by a defect in the nonreceptor tyrosine kinase Btk.[1] However, only about 80% of patients with agammaglobulinemia with markedly reduced numbers of B-lymphocytes in the peripheral blood are boys with a mutation in their *BTK* gene. In addition, 10% of patients with this phenotype are girls.[2] Therefore, a search was started for autosomal recessive disorders which result in an XLA-like phenotype. In this way, immunoglobulin (Ig) μ heavy chain deficiency[3] and λ14.1 pseudo light chain deficiency[4] were found to be the defects in some of the patients.

Normal B-cell differentiation in bone marrow is a tightly regulated process. It can be studied by analysing the sequential appearance and disappearance of intracellular and cell surface markers (Table 1).[5-7] Early B-cell progenitors express CD34, and subsequently B-cell specific markers like CD10 and CD19 are expressed as well as terminal deoxynucleotidyl transferase (TdT), which is necessary for nucleotide insertion during Ig gene rearrangement processes. Then cytoplasmic Igμ (Cylgμ) and later surface membrane Igμ (Smlgμ) appear. Smlgμ is first expressed as part of the pre-B-cell receptor (preBCR) on the cell surface (Igμ with pseudo light chain), and later on as IgM molecule (Igμ with Igκ or Igλ light chains) in both cases with CD79a and CD79b (Igα and Igβ) as the anchoring and signaling molecules. Abnormal B-cell development is reflected by the disturbance of the relative frequencies or even absence of particular precursor-B-cell subpopulations in bone marrow.[6,7]

We present a girl with agammaglobulinemia and absence of blood B-lymphocytes. We compared the findings in this patient with those in patients with known defects, and present a plan for unraveling the molecular defect in this girl.

PATIENT

The patient, a girl now aged 11 years, is the only child of healthy first cousin Turkish parents. Noteworthy are her slight facial dysmorphism, eczema, short stature (3rd percentile), bronchial hyperreactivity, Wolf-Parkinson-Wight syndrome and mild subvalvular aortic stenosis.

She was admitted directly after birth with wet lung and feeding problems. Consecutive white blood cell counts showed that the absolute neutrophil count decreased from 4.2 to 0.8 x 10^9/l in the first week of life, but this did not attract attention at that time. At two months of age, she was readmitted for feeding problems, failure to thrive, and severe neutropenia (<0.01 x 10^9/l).

She was referred with agammaglobulinemia, absent blood B-lymphocytes, and concomitant neutropenia (then 0.2 x 10^9/l) at nine months of age. After the onset of

Ig replacement therapy her neutropenia slowly improved (now around 1.0 x 10^9/l).

At six years of age, she suffered from an episode of diarrhea and weight loss caused by Giardia Lamblia infection, which was treated successfully with tinidazole. Osteomyelitis of the right distal tibia caused by *S. aureus* was treated successfully with intravenous flucloxacillin.

IMMUNOLOGICAL INVESTIGATIONS

CD19+ B-lymphocytes were never detected in peripheral blood (<1% of lymphocytes, < 0.01 x 10^9/l). The absolute counts of CD3+ T-lymphocytes (1.9 x 10^9/l), CD4+ helper T-lymphocytes (1.2 x 10^9 /l), CD8+ suppressor/cytotoxic T-lymphocytes (0.61 x 10^9/l), and CD16+ and/or 56+ /CD3- NK-cells (0.15 x 10^9 /l) were normal for age.[8]

At 5 months of age, IgG was 0.9 g/l, IgM 0.1 g/l, and IgA 0.1 g/l. After initiation of Ig replacement therapy, IgM and IgA remained undetectable.

Detailed immunophenotyping of bone marrow with triple labelings (see also Table 1) showed that 20% of mononuclear cells (MNC) were B-lineage cells (CD19+ cells; Figure 1a). 2% of MNC were CD34+/TdT+/CD19+ and CD10+/TdT+/CD19+ early precursor-B-cells (Figures 1a and 1b). 13% of MNC were CyIgμ+/SmIgM-/CD19+ pre-B-cells (Figure 1c). More mature B-cells (immature B) were low but present, and mainly had the CyIgμ+/SmIgM+/CD19+ immunophenotype (Figure 1c). As expected, cytoplasmic IgM, IgG and IgA containing plasma cells were completely absent.

Chromosomal analysis of the patient showed a normal 46XX karyotype and uniparental disomy was not present. The mother showed random X-inactivation of her B-lymphocytes. Both parents were healthy, and had normal serum Ig. Analysis of the *BTK* gene did not reveal any abnormalities (data not shown; kindly performed by R. Brooimans, Wilhelmina Children's Hospital, Utrecht).

DISCUSSION

This patient shows a phenotype that is "typical" for XLA (Btk deficiency). However, she is a girl, and chromosomal abnormalities that could explain XLA in a girl, like Turner's syndrome or uniparental disomy, were not present.[9] Her mother was not a carrier of XLA, nor was her father an XLA-patient. Not surprisingly, her *BTK* gene was found to be completely normal. Bone marrow of Igμ heavy chain deficient patients[3] shows a marked decrease in CyIgμ+ pre-B-cells that is not found in our patient. Bone marrow of the one patient described with λ14.1 pseudo light chain deficiency shows an even earlier developmental arrest, with most CD19+ cells being CD34+ precursor-B-cells.[4] The patient with the earliest defect in

TABLE 1. Model of human B-cell development based on the literature [refs. 6,7]

Stem cell	*Pro-B*	*Pre-B I*	*Large pre-B II*	*Small pre-B II*	*Immature B*	*Mature B*
CD34+	CD34+	CD34+				
H germline	H germline	DJ_H	$V_H DJ_H$	$V_H DJ_H$	$V_H DJ_H$	$V_H DJ_H$
L germline	L germline	L germline	L germline	$V_L J_L$	$V_L J_L$	$V_L J_L$
	ψL + (VpreB/λ14.1)	ψL + (VpreB/λ14.1)	ψL + (VpreB/λ14.1)			
	TdT+	TdT+				
	CyCD79a+ (Igα)	CyCD79a+ (Igα)	CyCD79a+ (Igα)			
	$CD10_{lo}$	CD10+	CD10+	CyCD79a+ (Igα)		
		CyCD79b+ (Igβ)	CyCD79b+ (Igβ)	CD10+	CD10+	
		CD19+	CD19+	CyCD79b+ (Igβ)		
			CyIgμ+	CD19+	CD19+	CD19+
			PreBCR+	CyIgμ+		
				PreBCR+		
					BCR/SmIgM+	BCR/SmIgM+
						BCR/SmIgD+

Abbreviations: H = heavy chain gene; L = light chain gene; ψL = pseudo light chain (composed of VpreB and λ14.1); Cy = cytoplasmic; (pre) BCR = (pre) B-cell receptor; Sm = surface membrane.

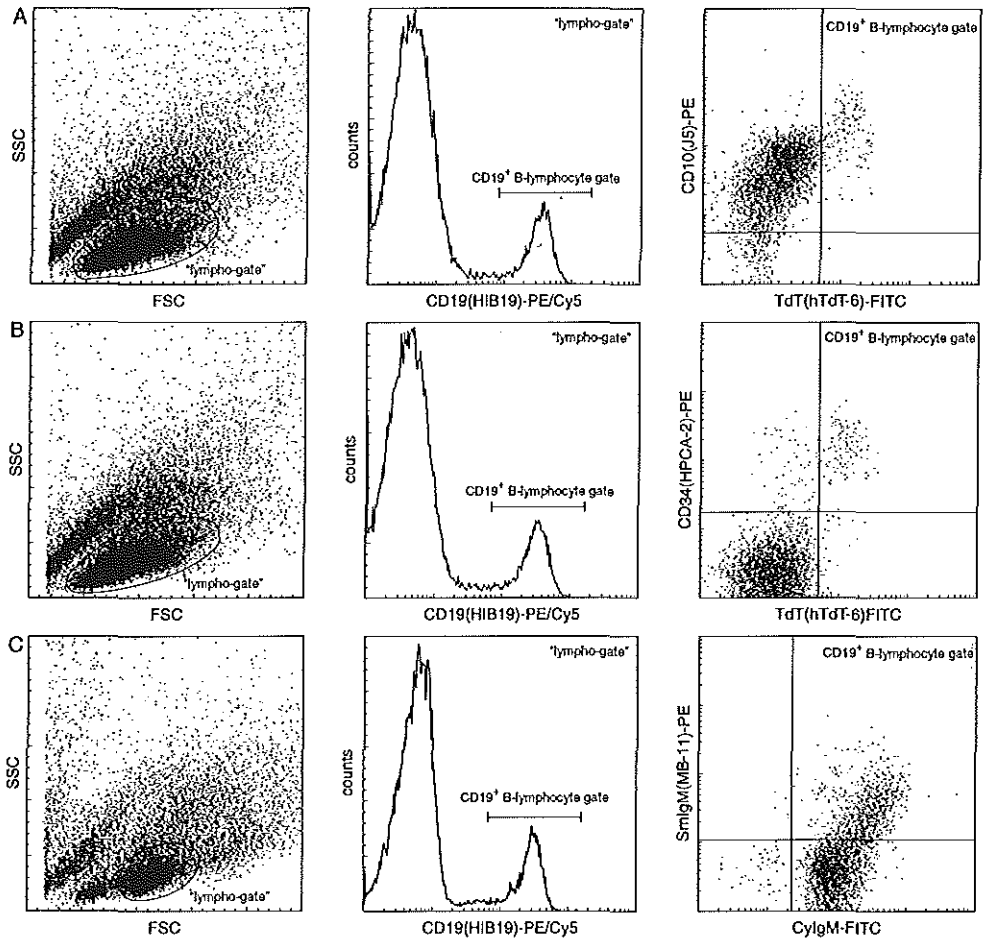


Figure 1. Immunophenotyping of bone marrow cells. Bone marrow mononuclear cells were stained for CD10 and CD19 (*A*) or CD34 and CD19 (*B*), followed by intracellular staining for TdT after permeabilization of the cells with FACS Lysing Solution (Becton Dickinson, San Jose, CA). The third triple labeling (*C*) was performed by staining for CD19 and SmIgμ, followed by intracellular Igμ (CyIgμ) staining, after permeabilization with IntraPrep (Immunotech, Marseille, France). All three triple labelings were evaluated via two consecutive gating steps, i.e. gating on the "lympho-gate" (left panels) based on light scatter characteristics, and subsequent gating on CD19 positivity (middle panels). These analyses showed that the vast majority of CD19+ precursor-B-cells are positive for CD10, but negative for the more immature markers CD34 and TdT. Furrthermore, most precursor-B-cells expressed CyIgμ and a small part also expressed SmIgμ.The combined data indicate that the precursor-B-cell compartment in bone marrow in this patient mainly consists of more mature precursor-B-cells, whereas no mature B-lymphocytes are found in peripheral blood. This implies that the differentiation blockade probably occurs just before the early B-lymphocyte stage.

B-cell development - of which the molecular defect is as yet unidentified - shows complete absence of Cylgμ, and very low levels of CD19+ cells in bone marrow.[6,10] Both bone marrow immunophenotypes are not comparable to the immunophenotype found in our patient. So, Btk-deficiency has been ruled out, and Igμ heavy chain deficiency, λ14.1 pseudo light chain deficiency, or the above mentioned early pro-B-cell blockage are not likely explanations for the B-lymphocyte deficiency in this girl.

Other disorders that result in agammaglobulinemia with a selective B-lymphocyte deficiency in humans have not been described as yet. However, several mouse models have been created in which genes have been knocked out that are supposed to be important in B-cell development. Some of them show a phenotype that is reminiscent of the "typical" XLA phenotype.[7,11] In order to find the molecular defect in patients like the one we describe here, it might be useful to screen for defects that have already been engineered in the mouse. Our patient only has a B-cell defect in the presence of a completely normal T-cell compartment. Therefore, defects in molecules that are indispensable for common lymphoid progenitors or that have an important role in T-cell development as well as in B-cell development are unlikely. B-cell specific molecules like CD79a and CD79b are interesting candidates, as are tyrosine kinases that are linked to the Btk-pathway.[7,11,12]

We propose the following plan for future research towards the underlying molecular defect in this girl:
1. Determine the integrity of the genes for Igμ and pseudo light chain (VpreB and λ14.1), because it is not yet fully clear whether genotype and phenotype are closely related in these disorders.
2. Perform more detailed immunophenotyping of the patient's bone marrow, including expression of CD79a and CD79b, to determine the exact position of the arrest in B-cell development.
3. Examine the integrity of other genes, including the *CD79A*, *CD79B*, *PAX5*, *SYK*, and possibly *SOX4* and *BLK* genes, dependent on the results of the additional immunophenotyping of the bone marrow and comparison of these results with those from the relevant knock-out mice.

If we succeed in unraveling the molecular defect in this girl, this will not only help her family (prenatal diagnosis and identification of carriers), but it will also lead to a further understanding of normal and abnormal human B-lymphocyte development.

REFERENCES

1 Rosen FS, Wedgwood RJP, Eibl M, Fischer A, Aiuti F, Notarangelo L, Kishimoto T, Resnick IB, Hammarstrom L, Seger R, Chapel H, Thompson RA, Cooper MD, Geha RS, Good RA, Waldmann TA. Primary immunodeficiency diseases. WHO Scientific Group. *Clin Exp Immunol* 1997;109(S1):1-28.
2 Conley ME. Autosomal recessive agammaglobulinemia. In: Ochs HD, Smith CIE, Puck JM (eds). *Primary immunodeficiency diseases. A molecular and genetic approach.* New York, Oxford University

Press, 1999, pp. 285-291.
3 Yel L, Minegishi Y, Coustan-Smith E, Buckley RH, Trübel H, Pachman LM, Kitchingman GR, Campana D, Rohrer J, Conley ME. Mutations in the mu heavy chain gene in patients with agammaglobulinemia. *N Eng J Med* 1996;335:1486-1493.
4 Minegishi Y, Coustan-Smith E, Wang Y-H, Cooper MD, Campana D, Conley ME. Mutations in the human *λ5/14.1* gene result in B cell deficiency and agammaglobulinemia. *J Exp Med* 1998;187:71-77.
5 Ghia P, Ten Boekel E, Sanz E, De la Hera A, Rolink A, Melchers F. Ordering of human bone marrow B lymphocyte precursors by single-cell polymerase chain reaction analyses of the rearrangement status of the immunoglobulin H and L chain gene loci. *J Exp Med* 1996;184:2217-2229.
6 Meffre E, LeDeist F, De Saint-Basile G, Deville A, Fougereau M, Fischer A, Schiff C. A non-XLA primary deficiency causes the earliest known defect of B cell differentiation in humans: a comparison with an XLA case. *Immunol Letters* 1997;57:93-99.
7 Burrows PD, Cooper MD. B cell development and differentiation. *Curr Opin Immunol* 1997;9:239-244.
8 Comans-Bitter WM, de Groot R, van den Beemd R, Neijens HJ, Hop WCJ, Groeneveld K, Hooijkaas H, van Dongen JJM. Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. *J Pediatr* 1997;130:388-393.
9 Conley ME, Sweinberg SK. Females with a disorder phenotypically identical to X-linked agammaglobulinemia. *J Clin Immunol* 1992;12:139-143.
10 Meffre E, LeDeist F, De Saint-Basile G, Deville A, Fougereau M, Fischer A, Schiff C. A human non-XLA immunodeficiency disease characterized by blockage of B cell development at an early proB cell stage. *J Clin Invest* 1996;98:1519-1526.
11 Fischer A, Malissen B. Natural and engineered disorders of lymphocyte development. *Science* 1998;280:237-243.
12 Satterthwaite A, Witte O. Genetic analysis of tyrosine kinase function in B cell development. *Annu Rev Immunol* 1996;14:131-154.

PART IV

DISCUSSION AND SUMMARY

CHAPTER 14

GENERAL DISCUSSION

GENERAL DISCUSSION

In the past two decades, knowledge of the human immune system has increased dramatically due to the expanding technical possibilities, of which flow cytometric immunophenotyping of leukocytes is a prominent example. Around 25 years ago, T-lymphocytes were defined by their capability of forming rosettes with sheep erythrocytes, and B-lymphocytes by the presence of immunoglobulins on their surface membrane.[1,2] Nowadays, more than 100 different cell surface and intracellular markers can be analyzed by detailed multiparameter flow cytometry to define T-lymphocytes, B-lymphocytes, NK-cells and their various subpopulations.[3] These findings can be coupled to functional characteristics of the different subsets, many of which can also be determined by flow cytometry.[4]

Because adult blood is more easily available than infant blood, most studies on flow cytometric immunophenotyping of leukocytes have been performed in adults. The technical experience[5] and reference values[6] collected with adult blood samples, were applied to blood samples from neonates and infants to investigate normal lymphocyte development in the first years of life [7] as well as the pathophysiology of congenital and acquired immunodeficiencies.[8]

However, several problems and pitfalls are encountered when analyzing lymphocytes in pediatric samples (Chapters 4 and 5), like the limited amount of available blood in neonates and infants, the presence of normoblasts and lysis-resistant erythrocytes in neonatal cord blood, the variability in staining patterns of some fluorochrome-conjugated antibodies (McAb) combinations in neonates, and the higher lymphocyte counts in neonates and infants, which render comparative lymphocyte studies between neonates, infants, and adults less informative if relative frequencies of lymphocytes are used.

Technical aspects of immunophenotyping of lymphocytes in children

This thesis shows that it is possible to overcome the above mentioned problems and pitfalls and to acquire accurate absolute counts of lymphocyte subpopulations in neonates and infants.

When microassays are used for immunological studies, the limitations in the available volume of blood in neonates and infants is no longer a problem. We showed that it is possible to develop a reliable microassay for immunophenotyping of lymphocytes in neonates and infants (Chapter 3). It is desirable, and potentially possible, to develop other microassays for immunological investigations in children in the future, e.g. for studies on lymphocyte function.

The normoblasts and unlysed erythrocytes within the flow cytometric "lymphogate" of neonatal samples can be identified using the cluster of differentiation (CD)71/GpA/CD45 triple immunostaining (Chapter 3): the transferrin receptor CD71 is expressed by erythroid precursors, glycophorin A (GpA) is expressed by all erythroid cells, and CD45 is expressed by all leukocytes. After identification of these normoblasts and unlysed erythrocytes, a correction for their presence can be made.

This technique can also be used for rapid prenatal diagnosis of congenital immunodeficiencies by flow cytometric immunophenotyping in fetal blood samples, which contain a very large amount of normoblasts.[9]

The importance of using absolute counts instead of relative frequencies was illustrated by our finding that the absolute counts of CD45RO+ T-lymphocytes are similar in healthy neonates, infants, and adults, despite higher relative frequencies of CD45RO+ T-lymphocytes in adults (Chapter 7) and that the high percentage of T-cell receptor (TCR)γδ+ T-lymphocytes in the two siblings with abnormal cell-mediated immunity was due to TCRαβ+ T-lymphocytopenia with normal absolute counts of TCRγδ+ T-lymphocytes (Chapter 11).

Using detailed multiparameter flow cytometry, we and others showed that immature "thymocyte-like" T-cells are *not* present in the neonatal circulation. These cells were assumed to be present, based on single CD1, CD4, CD8, and CD71 immunostainings in the past.[10-12] Now, it has become clear that CD71+ cells are mainly normoblasts (not thymocytes) (Chapters 4 and 5), that CD1+ cells represent CD1c+ B-lymphocytes and not CD1a+ thymocytes (Chapters 4 and 7 and ref 13), and that CD4+/CD8+ "double-positive" thymocytes are not present (Chapters 4 and 7 and ref 14), but that the high CD8+ count is due to the high CD8+/CD3- NK-cell count (Chapters 4 and 7) and that CD4+/CD3- cells are in fact CD14+ small monocytes, not immature T-cells (Chapter 4).

We (Chapters 5, 6, and 7) and others[15] have shown differences in lymphocyte subpopulations between neonates, infants and adults. This implies that the available adult reference values cannot be used in children. In our studies, this was clearly illustrated by the analysis of V_γ and V_δ gene usage in the two siblings with TCRαβ+ T-lymphocytopenia (Chapter 11), and by the analysis of NK-cell function in the children with loss of the B73.1 (CD16) epitope (Chapter 10). In the near future, reference values for all pediatric age groups will have to be established for relative frequencies as well as absolute counts of specific lymphocyte subpopulations (e.g. CD45RA+ and CD45RO+ T-lymphocyte subpopulations), and also for functional tests like lymphocyte proliferation assays, and analyses of cytokine production by different subsets.

Immunophenotyping of lymphocytes in healthy neonates and infants

With detailed multiparameter flow cytometry, reference values of pediatric

lymphocytes can be accurately determined in cross-sectional studies.[16] However, external factors that influence lymphocyte development, like exposure to specific antigens, will not happen at exactly the same time-points in each child. Therefore, longitudinal follow-up studies of individual children will give additional information on lymphocyte development in the first years of life.

In this thesis, we show that trends in lymphocyte subpopulation changes found in populations in cross-sectional studies[16] are confirmed when following individual children longitudinally: T- and B-lymphocyte absolute counts increase at one and six weeks of age, respectively, whereas NK-cell counts show a sharp decline directly after birth. The latter is mainly due to a decline in CD8+ NK-cells. The high CD4/CD8 ratio in infants is mainly due to the very high CD4+ T-lymphocyte counts (Chapters 6 and 7).

The comparison of absolute counts of lymphocyte subpopulations gave new insight into lymphocyte development in infants, when compared to previous studies which only used relative frequencies.[6,9,17] Large amounts of naive "untriggered" cells (e.g. CD45RA+ T-lymphocytes) are present in neonates, and further increase during the first year of life, whereas "memory" cells (CD45RO+ T-lymphocytes) are present in similar amounts in neonates, infants, and adults, a finding that will be missed if only relative frequencies are considered.

These findings alter the perspective on the development of the infant immune system considerably: neonates and infants do not lack mature cells, but they have large amounts of "untriggered" naive cells, probably being ready to be primed in primary immune responses. However, the "memory" T-lymphocytes in neonates and infants did show differences in comparison with adult cells: the fluorescence intensity of CD45RO staining was lower in neonates and infants than in adults (i.e. fewer molecules were present per cell), and more neonatal and infant cells carried CD38 on their surface, probably as a sign of greater metabolic need due to continuous encounters with antigen in primary immune responses (Chapter 7). Functional studies are needed to assess whether the seemly "mature" cell populations in neonates and infants that show phenotypic differences with their adult counterparts are truly mature, or whether they harbor some kind of "functional immaturity" that is gradually overcome during maturation processes in the first few years of life.

Immunophenotyping of lymphocytes in immunodeficient children

Knowledge of lymphocyte subpopulations in healthy children enables the interpretation of results obtained in immunodeficient children. Despite the rapid developments in genetic research,[18] immunophenotyping remains an important tool in the diagnosis of congenital immunodeficiency states in children because it is reliable, rapid and easy-to-use. It is also important for follow-up of children with acquired immunodeficiency states, e.g. after bone marrow transplantation. In this thesis, this is illustrated by immunophenotyping studies in patients with a variety of

underlying conditions.

We (Chapter 9) and others[8,9,19] found that the reconstitution of lymphocyte subpopulations after bone marrow transplantation does not resemble normal ontogeny as far as T-lymphocytes are concerned. Especially the very slow reconstitution of CD45RA+ cells does not resemble T-cell development in the first year of life. B-lymphocyte reconstitution does seem to follow normal ontogeny with high values being reached after six months posttransplant. When age-matched reference values are used to define reconstitution, it becomes clear that CD4+ T-lymphocyte absolute counts recover slowly in children, as has been described in adults.[19] Unexpectedly, the reconstitution of CD4+ T-lymphocyte absolute counts is faster in children with CMV-reactivation (Chapter 9).

Immunophenotyping of lymphocytes is essential for focussing the molecular diagnostic studies in congenital immunodeficiency diseases in order to prevent that too many genes have to be analysed for the presence of mutations. In this thesis, this is illustrated by the B73.1 (CD16) epitope loss in the children with $F_c\gamma$RIIIA48-H/H homozygosity (Chapter 10), and by the absence of CD40 ligand expression on activated T-lymphocytes in the patients with X-linked hyper IgM syndrome (Chapter 12). Furthermore, immunophenotyping can reveal the presence of an immune disturbance of which the molecular defect cannot be defined as yet, as is illustrated in the two siblings with TCR$\alpha\beta$+ T-lymphocytopenia (Chapter 11). It is important to realize, however, that an immune disturbance can be present without detectable abnormalities in immunophenotyping of lymphocytes.

Immunophenotyping of bone marrow cells can direct the search towards the underlying molecular defect in B-cell immunodeficiencies by identifying the developmental block, as is shown in the girl with agammaglobulinemia and absent blood B-lymphocytes (Chapter 13).

Even if the molecular defect of an immunodeficiency is already established, immunophenoptyping of lymphocytes enables pathogenetic studies of the defect by analyzing subsets according to e.g. their status of activation, expression of adhesion molecules, or intracellular expression of cytokines.[18]

In conclusion, immunophenotyping of lymphocytes proofs to be a valuable tool for pediatricians in the care of immunodeficient children for many years to come.

REFERENCES

1 Sandahl Christiansen J, Osther K, Peitersen B, Bach-Mortensen N. B, T and null lymphocytes in newborn infants and their mothers. *Acta Paediatr Scand* 1976;65:425-428.

2 Ugazio AG, Marcioni AF, Astaldi Ajr, Burgio GR. Peripheral blood B lymphocytes in infancy and childhood. *Acta Paediatr Scand* 1974;63:205-208.

3 Kishimoto T, Kikutani H, Von dem Borne AEGKr, Goyert SM, Mason DY, Miyasaka M, Moretta L, Okumura K, Shaw S, Springer TA, Sugamura K, Zola H (Eds.). Leukocyte typing VI. New York, Garland Publishing, 1997.

4 North ME, Ivory K, Funauchi M, Webster AD, Lane AC, Farrant J. Intracellular cytokine production by human CD4+ and CD8+ T cells from normal and immunodeficient donors using directly conjugated anti-cytokine antibodies and three-colour flow cytometry. *Clin Exp Immunol* 1996;105:517-522.
5 Fleisher TA. Immunophenotyping of lymphocytes by flow cytometry. *Immunol Allergy Clin* 1994;14:225-240.
6 Groeneveld K, Comans-Bitter WM, van den Beemd MWM, et al. Blood T-cell subsets in health and disease. *J Int Fed Clin Chem* 1994;6:84-94.
7 Andersson U, Bird G, Britton S. Cellular mechanisms of restricted immunoglobulin formation in the human neonate. *Eur J Immunol* 1980;10:888-94.
8 Kroczek RA, Graf D, Brugnoni D, Giliani S, Korthauer U, Ugazio A, Senter G, Mages HW, Villa A, Notarangelo LD. Defective expression of CD40 ligand on T cells causes X-linked immunodeficiency with hyper-IgM (HIGM1). *Immun Rev* 1994;138:39-59.
9 Oski FA. The erythrocyte and its disorders. In: Nathan DG, Oski FA (eds) *Hematology of infancy and childhood.* Saunders, Philadelphia, 1993, pp18-43.
10 Foa R, Giubellino MC, Fierro MT, Lusso P, Ferrando ML. Immature T lymphocytes in human cord blood identified by monoclonal antibodies: a model for the study of the diffentiation pathway of T cells in humans. *Cell Immunol* 1984;89:194-201.
11 Gerli R, Rambotti P, Cernetti C, Velardi A, Spinozzi F, Tabilio A, Martelli MF, Grignani F, Davis S. A mature thymocyte-like phenotypic pattern on human cord circulating T-lymphoid cells. *J Clin Immunol* 1984;4:461-468.
12 Griffiths-Chu S, Patterson JAK, Berger CL, Edelson RL, Chu AC. Characterization of immature T cell subpopulations in neonatal blood. *Blood* 1984;64:296-300.
13 Durandy A, Thuillier L, Forveille M, Fischer A. Phenotypic and functional characteristics of human newborns' B lymphocytes. *J Immunol* 1990;144:60-65.
14 Reason DC, Ebisawa M, Saito H, Nagakura T, Iikura Y. Human cord blood lymphocytes do not simultaneously express CD4 and CD8 cell surface markers. *Biol Neonate* 1990;58:87-90.
15 Erkeller-Yuksel FM, Deneys V, Yuksel B, Hannet I, Hulstaert F, Hamilton C, Mackinnon H, Turner Stokes L, Munhyeshuli V, Vanlangendonck F, De Bruyère FM, Bach BA, Lydyard PM. Age-related changes in human blood lymphocyte subpopulations. *J Pediatr* 1991;120:216-22.
16 Comans-Bitter WM, de Groot R, van den Beemd R, Neijens HJ, Hop WCJ, Groeneveld K, Hooijkaas H, van Dongen JJM. Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. *J Pediatr* 1997;130:388-93.
17 Kingsley G, Pitzalis C, Waugh AP, Panayi GS. Correlation of immunoregulatory function with cell phenotype in cord blood lymphocytes. *Clin exp Immunol* 1988;73:40-45.
18 Rosen FS, Wedgwood RJP, Eibl M, Fischer A, Aiuti F, Notarangelo L, Kishimoto T, Resnick IB, Hammarstrom L, Seger R, Chapel H, Thompson RA, Cooper MD, Geha RS, Good RA, Waldmann TA. Primary immunodeficiency diseases. Report of a WHO Scientific Group. *Clin Exp Immunol* 1997;109(S1):1-28.
19 Storek J, Witherspoon RP, Storb R. T cell reconstitution after bone marrow transplantation into adult patients does not resemble T cell development in early life. *Bone Marrow Transplant* 1995; 16: 413-425.

SUMMARY

At birth, the "naive" immune system is suddenly challenged with many environmental antigens. In this process, T-lymphocytes and B-lymphocytes play a central role. The "naive" immune system gradually builds up a pool of competent "memory" cells during the first two years of life by its encounters with antigens. It is to be expected that this process is reflected in the distribution of lymphocyte subpopulations, and that such processes will be disturbed in certain immunodeficiency diseases.

The aim of this thesis was to study development of lymphocytes with immunophenotyping in healthy as well as immunodeficient children. This information can lead to a better understanding of the immaturity of the immune system in infants and their higher susceptibility to infections. Also, the improved knowledge of the normal development of the immune system can lead to a better interpretation of results obtained in studies of immunodeficient patients. The technical developments in the past decade have greatly improved the possibilities for such studies: nowadays, rapid and accurate multiparameter flow cytometric immunophenotyping of lymphocytes enables detailed studies of lymphocyte subpopulations. By incubating the unprocessed blood sample with monoclonal antibodies (McAb) coupled to a fluorochrome, and analysing after osmotic lysis of the erythrocytes - the lysed whole blood technique - differential loss of subpopulations and aspecific binding of McAb are minimized (Chapter 2).

However, several problems and pitfalls are encountered when immunophenotyping of lymhocytes is undertaken *in children* (Chapter 4). Firstly, the limited amount of blood available hampers the possibilities for detailed studies in smaller children. This can be overcome by developing microassays (Chapter 3). Secondly, the analysis of lymphocytes within the flow cytometric "lympho-gate" - defined on the basis of size and cellular characteristics of the cells - can be biased by contamination of this "lympho-gate" with erythroid cells which can be present in large numbers in neonatal cord blood (median around 30% in preterm neonates and 10% in term neonates). This leads to underestimation of the relative frequencies of lymphocyte subpopulations. Also, neonatal erythrocytes are relatively resistant to osmotic lysis, and are sometimes large enough to be located within the "lympho-gate" as well (>1% in about half of term neonates and in most preterm neonates). This can be overcome by using the cluster of differentiation (CD)71/GpA/CD45 triple immunostaining to identify normoblasts (CD71+/GpA+/CD45- cells) and unlysed erythrocytes (CD71-/GpA+/CD45- cells) within the "lympho-gate", and to correct for their presence by calculating the correct percentage of lymphocytes within the "lympho-gate" (Chapter 3).

In the past, the limited technical possibilities lead to erroneous interpretation of

immunophenotyping results, e.g. the assumption that immature "thymocyte-like" T-cells are present in the neonatal circulation. Those past conclusions have now been adapted gradually with the aid of more detailed multiparameter analysis of lymphocyte subpopulations (Chapter 4).

An additional problem is encountered with immunophenotyping of lymphocytes in neonates: some McAb like CD45RA and CD45RO show greater overlap in positive and negative populations than in adults, which renders the interpretation of results difficult. We preferred the use of phycoerythrin (PE) conjugated CD45RA and CD45RO McAb, because the greater staining intensity of the PE fluorochrome enabled us to define the quadrant settings more accurately (Chapter 5).

Healthy children

After addressing the above described technical problems, we were able to obtain accurate absolute counts of blood lymphocyte subpopulations in neonates and infants. This was important, because total lymphocyte counts per ml of blood are essentially higher in neonates and infants than in adults. Therefore, differences in the relative frequencies of lymphocyte subpopulations do not show the differences in the actual sizes (i.e. the absolute counts) of these subpopulations (Chapters 4-7). This is illustrated clearly with our findings in neonatal cord blood samples: we found that "mature" lymphocyte subsets - CD45RO+ T-lymphocytes, CD1c- and CD5- B-lymphocytes - are *similar* in size in neonates and adults. "Naive" subsets - CD45RA+ T-lymphocytes, CD1c+ and CD5+ B-lymphocytes - are larger in neonates than in adults: a large pool of naive as yet "untriggered" cells seems to be stand-by for participation in primary immune responses. This was not the case in some preterm neonates, which might explain their greater susceptibility to infections (Chapter 5).

Age-matched reference values have been determined in cross-sectional studies, but patients are generally followed longitudinally. Also, changes in lymphocyte subpopulations will not occur at exactly the same time-points in all children, and will therefore be leveled off in cross-sectional studies. Therefore, we performed a longitudinal study of lymphocyte subpopulations in healthy infants followed from birth to one year of age. We found that T- and B-lymphocytes increased at one and six weeks of age respectively, whereas NK-cells showed a sharp decline directly after birth. These results confirmed the findings we reported before in a cross-sectional study. The high CD4/CD8 ratio was mainly caused by very high CD4+ T-lymphocyte counts (Chapter 6). During the first year of life, the "naive" CD45RA+ T-lymphocyte subset increased in number; the "memory" CD45RO+ T-lymphocytes remained similar in size, the "mature" CD1c- and CD5- B-lymphocytes increased. We found no indication for the presence of immature "thymocyte-like" T-cells, nor for the presence of precursor-B-cells in the circulation. The functional maturation of T-lymphocytes in the periphery through triggering with antigen was reflected in the loss of CD38 expression and the increase of the intensity of expression of CD45RO per cell. The high NK-cell count at birth was

mainly accounted for by CD8+ NK-cells. The meaning of this finding is not clear. Interestingly, subpopulations of CD7-, CD38-, and CD45RO- NK-cells were present shortly after birth, whereas CD57+ NK-cells were absent, a phenotype which fits in with an activated state of these NK-cells. This finding can support the theory that NK-cells are engaged in the maintenance of pregnancy at the immunological interface in the placenta (Chapter 7).

Immunodeficient children

Immunophenotyping of lymphocytes plays an important role in the diagnosis and follow-up of primary and acquired immunodeficiencies. Enumeration of absolute counts of blood lymphocyte subpopulations enables diagnosis of congenital immunodeficiencies, follow-up of HIV-infection, and monitoring of immunological reconstitution after bone marrow transplantation. Also, in congenital immunodeficiencies the block in lymphocyte development can be determined by analysis of intracellular and surface antigen expression in blood and bone marrow cells (Chapter 8).

We studied twenty-two children who underwent allogeneic bone marrow transplantation (BMT) for a hematological malignancy, and compared the reconstitution of their lymphocyte subpopulations - defined as reaching the p_5 of age-matched reference values - to normal ontogeny. We found a relatively fast reconstitution of B-lymphocytes, with high values (>p_{95} of age-matched reference values) being reached from six months after BMT onwards, mimicking normal ontogeny. Unlike normal ontogeny, T-lymphocyte reconstitution was slow. The reconstitution of CD4+ helper T-lymphocytes was very slow (> 6 months after BMT), as was reported before in adults. Unexpectedly, CMV-reactivation enhanced CD4+ helper T-lymphocyte reconstitution. CD45RA+ T-lymphocyte absolute counts increased only slowly after BMT; CD45RO+ T-lymphocyte absolute counts remained similar during the entire follow-up period, despite the high relative frequency of CD45RO+ T-lymphocytes in the first three months after BMT. Like in adults, we found that a high percentage of T-lymphocytes was negative for the CD7 antigen in the first three months after BMT. However, CD7- T-lymphocyte absolute counts were similar during the entire follow-up period. These findings again illustrate the importance of using absolute counts instead of relative frequencies for investigating the development of lymphocyte subpopulations (Chapter 9).

In a child with recurrent - probably viral - respiratory tract infections and problems with BCG vaccination and infection with Epstein-Barr virus and varicella zoster virus, immunophenotyping of lymphocytes showed lack of staining with the CD16 (B73.1) epitope (CD16 is the Fcγ receptor type IIIa). CD3-/CD7+ NK-cells were present, however, and other CD16 McAb like 3G8 and CLBFcRgran1 did stain the patient's NK-cells. NK-cell function (K562 lysis and P815 ADCC) was normal as compared to an age-matched control, though lower than in adult controls. This CD16 (B73.1) epitope loss in the first extracellular domain of the CD16a molecule led to the finding of FcγRIIIA48-H/H homozygosity. The

importance of this finding is not yet clear, because the expected frequency of homozygotes is 6.4 per thousand Caucasians based on the allelic frequency found in healthy blood donors. No other homozygous individuals were identified until now (Chapter 10).

In several other immunodeficient patients, immunophenotyping proved to be useful for establishing the diagnosis or classification of their disease. In that way, the absence of CD40 ligand expression on activated T-lymphocytes could rapidly be shown in several patients with hyper IgM syndrome (Chapter 12), and the T-cell deficiency in two siblings with recurrent severe - mainly viral - infections could be further characterized (Chapter 11). Also, the exact position of the developmental blockage in B-cell differentiation could be determined in the bone marrow of a girl with agammaglobulinemia (Chapter 13). So, the current technical possibilities and available experience in flow cytometric immunophenotyping are very useful for the care of children with an immunodeficiency.

SAMENVATTING

Bij de geboorte wordt het "naieve" immuunsysteem plotseling geconfronteerd met de vele antigenen uit de omgeving. Hierbij spelen de T- en B-lymfocyten een centrale rol. Het "naieve" immuunsysteem bouwt gedurende de eerste twee levensjaren geleidelijk een verzameling competente "geheugen" cellen op door de ontmoetingen met antigenen. Het is te verwachten dat dit proces wordt weerspiegeld in de verdeling van de lymfocytensubpopulaties, en dat dergelijke processen gestoord zullen zijn bij bepaalde immunodeficiënties (afweerstoornissen).

Het doel van dit proefschrift was om met behulp van immunofenotypering de ontwikkeling van lymfocyten te bestuderen bij gezonde kinderen en kinderen met een afweerstoornis. De informatie verkregen uit een dergelijke studie kan leiden tot een beter begrip van de rijping van het immuunsysteem in jonge kinderen en van hun verhoogde gevoeligheid voor infecties. Daarnaast kan verbeterde kennis van de normale ontwikkeling van het immuunsysteem bijdragen aan de interpretatie van bevindingen uit studies bij immunodeficiënte patiënten. De technische ontwikkelingen van de afgelopen decade hebben de mogelijkheden voor dergelijke studies sterk verbeterd: tegenwoordig kan snel en nauwkeurig onderzoek van de lymfocytensubpopulaties plaatsvinden met behulp van multiparameter flowcytometrische immunofenotypering. Door het onbewerkte bloedmonster te incuberen met monoclonale antistoffen (McAb) die direct aan een fluorochroom zijn geconjugeerd, en te analyseren na osmotische lysis van de erytrocyten - de "gelyseerde vol bloed methode" - kan differentieel verlies van subpopulaties en aspecifieke binding van antistoffen veelal worden voorkomen (Hoofdstuk 2).

Echter, als immunofenotypering van lymfocyten *bij kinderen* wordt toegepast, komen diverse problemen en valkuilen aan het licht (Hoofdstuk 4). Allereerst bemoeilijkt het beperkte bloedvolume de mogelijkheden voor gedetailleerde studies bij kinderen. Met de ontwikkeling van micro-bepalingen kan dit probleem worden opgelost (Hoofdstuk 3). Ten tweede kan de analyse van lymfocyten binnen de flowcytometrische "lymfo-gate" - gedefinieerd op basis van de celgrootte en de onregelmatigheid van de cellen - worden verstoord door verontreiniging van deze "lymfo-gate" met erytroide cellen die in grote aantallen in neonataal navelstrengbloed aanwezig kunnen zijn (mediaan rond 30% bij prematuren, en rond 10% bij à terme neonaten). Dit leidt tot onderschatting van de relatieve frequenties van de lymfocytensubpopulaties. Daarnaast zijn neonatale erytrocyten relatief resistent voor osmotische lysis, en daarbij soms groot genoeg om ook binnen de "lymfo-gate" te vallen (>1% van de "lymfo-gate" bij ongeveer de helft van de à terme neonaten, en bij de meeste prematuren). Dit probleem kan worden opgelost door de differentiatie-cluster (CD)71/GpA/CD45 drievoudige labeling te gebruiken ter identificatie van normoblasten (CD71+/GpA+/CD45- cellen) en

ongelyseerde erytrocyten (CD71-/GpA+/CD45- cellen) binnen de "lymfo-gate", en door daarmee het correcte percentage lymfocyten binnen de "lymfo-gate" te berekenen (Hoofdstuk 3).

In het verleden hebben de beperkte technische mogelijkheden geleid tot onjuiste interpretaties van immunofenotyperingsresultaten, bijvoorbeeld tot de aanname dat onrijpe "thymocyt-achtige" T-cellen aanwezig zouden zijn in de neonatale circulatie. Dergelijke conclusies uit het verleden zijn geleidelijk aangepast met behulp van verfijnde analysemogelijkheden van lymfocytensubpopulaties met multiparameter flowcytometrie (Hoofdstuk 4).

Een extra probleem bij immunofenotypering van lymfocyten bij neonaten is het feit dat sommige McAb zoals CD45RA en CD45RO een grotere overlap tussen positieve en negatieve populaties laten zien dan bij volwassenen, wat de interpretatie van studieresultaten lastig maakt. Wij gaven de voorkeur aan het gebruik van phycoerythrine (PE) geconjugeerde CD45RA en CD45RO McAb, omdat de hogere kleuringsintensiteit van het PE-fluorochroom het ons mogelijk maakte om nauwkeuriger de grenzen tussen positieve en negatieve populaties vast te stellen (Hoofdstuk 5).

Gezonde kinderen

Na het aanpakken van de hierboven beschreven problemen waren wij in staat om de absolute aantallen van lymfocytensubpopulaties bij neonaten en jonge kinderen nauwkeurig te bepalen. Dit was belangrijk omdat het totaal aantal lymfocyten per ml bloed bij neonaten en jonge kinderen hoger is dan bij volwassenen. Dat betekent dat verschillen tussen relatieve frequenties van lymfocytensubpopulaties niet de verschillen in daadwerkelijke grootte (d.w.z. absolute aantallen) van deze subpopulaties laten zien (Hoofdstukken 4-7). Dit wordt duidelijk geïllustreerd door onze bevindingen in neonataal navelstrengbloed: we vonden dat "rijpe" lymfocytensubpopulaties - CD45RO+ T-lymfocyten, CD1c- en CD5- B-lymfocyten - een *vergelijkbare* grootte hadden bij neonaten en volwassenen."Naieve" subpopulaties - CD45RA+ T-lymfocyten, CD1c+ en CD5+ B-lymfocyten -waren in veel grotere aantallen aanwezig bij neonaten dan bij volwassenen: een grote verzameling naieve nog niet "getriggerde" cellen lijkt klaar te staan voor deelname aan primaire immuunreacties. Dit werd echter niet bij alle prematuren gevonden, wat hun grotere gevoeligheid voor infecties zou kunnen verklaren (Hoofdstuk 5).

Leeftijdsgerelateerde referentiewaarden worden veelal bepaald in transversale studies, terwijl patiënten longitudinaal worden vervolgd. Daarnaast zullen veranderingen in lymfocytensubpopulaties niet bij alle kinderen op exact dezelfde tijdstippen optreden, waardoor ze worden afgevlakt in transversale studies. Wij verrichtten daarom een longitudinale studie naar het verloop van de lymfocytensubpopulaties bij gezonde kinderen die werden vervolgd van hun geboorte tot de leeftijd van één jaar. We vonden dat T- en B-lymfocyten toenamen

in aantal vanaf de leeftijd van respectievelijk één en zes weken, terwijl de NK-cellen juist sterk daalden na de geboorte. Deze resultaten bevestigden onze bevindingen uit een transversale studie die we al eerder rapporteerden. De hoge CD4/CD8 ratio werd voornamelijk veroorzaakt door de zeer hoge absolute aantallen CD4+ T-lymfocyten (Hoofdstuk 6). Gedurende het eerste levensjaar stegen de "naieve" CD45RA+ T-lymfocyten in aantal; de "memory" CD45RO+ T-lymfocyten bleven gelijk in aantal, de "rijpe" CD1c- en CD5- B-lymfocyten stegen in aantal. We vonden geen aanwijzingen voor de aanwezigheid van onrijpe "thymocyt-achtige" T-cellen of voorloper B-cellen in de circulatie. De functionele rijping van T-lymfocyten in de periferie door antigene stimulatie werd weerspiegeld door het afnemen van de CD38 expressie en het toenemen van de intensiteit van de CD45RO expressie. De hoge aantallen NK-cellen bij de geboorte waren voor een groot deel CD8+ NK-cellen. De betekenis hiervan is niet duidelijk. Opvallend was de aanwezigheid van CD7-, CD38-, en CD45RO- NK-cellen kort na de geboorte, en de afwezigheid van CD57+ NK-cellen, een fenotype dat kan passen bij een geactiveerde status van deze NK-cellen. Deze bevinding kan de theorie ondersteunen dat deze ongebruikelijke NK-cellen actief zijn bij het behoud van de zwangerschap op het immunologische scheidingsvlak in de placenta (Hoofdstuk 7).

Kinderen met een afweerstoornis

Immunofenotypering van lymfocyten speelt een belangrijke rol bij de diagnostiek en het vervolgen van primaire en verkregen immunodeficiënties. Het nauwkeurig vaststellen van de absolute aantallen van de lymfocytensubpopulaties in het bloed maakt de diagnostiek mogelijk van congenitale immunodeficiënties, het vervolgen van HIV-infectie, en het monitoren van het immunologisch herstel na beenmergtransplantatie. Tevens kan bij congenitale immunodeficiënties de blokkade in de ontwikkeling van de lymfocyten worden vastgesteld door middel van analyse van de expressie van intracellulaire en oppervlakte moleculen van cellen in bloed en beenmerg (Hoofdstuk 8).

Wij bestudeerden het herstel van de lymfocytensubpopulaties - gedefiniëerd als het bereiken van de p_5 van leeftijdsgerelateerde normaalwaarden - bij tweeëntwintig kinderen die een allogene beenmergtransplantatie (BMT) ondergingen voor een hematologische maligniteit, en vergeleken de resultaten met de normale lymfoïde ontwikkeling. We vonden een relatief snel herstel van de aantallen B-lymfocyten, waarbij hoge waardes ($>p_{95}$ van leeftijdsgematchte normaalwaarden) werden bereikt vanaf zes maanden na BMT, zoals in de normale ontwikkeling ook gebeurt. In tegenstelling tot de normale ontwikkeling was het herstel van de aantallen T-lymfocyten traag. Het herstel van de aantallen CD4+ helper T-lymfocyten was zelfs zeer traag (> zes maanden na BMT), zoals al eerder voor volwassenen werd beschreven. Onverwachts vonden we een duidelijk positief effect van CMV-reactivatie op het herstel van de aantallen CD4+ helper T-lymfocyten. De aantallen CD45RA+ T-lymfocyten stegen slechts langzaam na BMT; de aantallen CD45RO+ T-lymfocyten bleven gedurende de gehele follow-up

periode gelijk, ondanks de hoge relatieve frequentie van CD45RO+ T-lymfocyten in de eerste drie maanden na BMT. Net als bij volwassenen vonden we dat een hoog percentage van de T-lymfocyten in de eerste drie maanden na BMT negatief was voor het CD7 molecuul. De absolute aantallen CD7- T-lymfocyten waren echter gedurende de gehele follow-up periode gelijk. Dit illustreert opnieuw het belang van het gebruik van absolute aantallen in plaats van relatieve frequenties bij onderzoek naar de ontwikkeling van lymfocytensubpopulaties (Hoofdstuk 9).

Bij een kind met recidiverende - waarschijnlijk virale - luchtweginfecties en problemen met BCG-vaccinatie en infectie met Epstein-Barr virus en varicella zoster virus toonde immunofenotypering van de lymfocyten een afwezigheid van het CD16 (B73.1) epitoop aan (CD16 is de Fcγ receptor type IIIa). CD3-/CD7+ NK-cellen waren echter wel aanwezig, en andere CD16 McAb zoals 3G8 en CLBFcRgran1 kleurden de NK-cellen van de patiënt wel aan. De NK-cel functie (K562 lysis en P815 ADCC) was normaal in vergelijking met een leeftijdsgerelateerde controle; overigens was dat wel lager dan bij volwassen controles. Dit CD16 (B73.1) epitoopverlies in het eerste extracellulaire domein van het CD16a molecuul leidde tot de ontdekking van FcγRIIIA48-H/H homozygotie in de patiënt. Het belang van deze bevinding is nog niet duidelijk, aangezien de verwachte frequentie van homozygoten 6.4 per duizend Caucasiërs is, gebaseerd op de gevonden allelfrequentie bij gezonde bloeddonoren. Tot nu toe werden nog geen additionele homozygote individuen geïdentificeerd (Hoofdstuk 10).

Ook bij diverse andere patiënten met een immunodeficiëntie bleek gedetailleerde immunofenotypering waardevol te zijn voor het stellen van de diagnose of voor het nauwkeurig definiëren van het ziektebeeld. Zo kon op snelle wijze de CD40 ligand deficiëntie worden aangetoond op geactiveerde T-lymfocyten bij patiënten met hyper IgM syndroom (Hoofdstuk 12), en kon het type T-cel deficiëntie nauwkeurig worden vastgesteld bij een broer en zus met ernstige recidiverende infecties van vooral virale oorsprong (Hoofdstuk 11).Tenslotte kon de precieze positie van de blokkade in de B-cel differentiatie worden bepaald in het beenmerg van een meisje met agammaglobulinemie (Hoofdstuk 13). Kortom, de huidige technische mogelijkheden en de beschikbaar gekomen kennis en ervaring op het gebied van flowcytometrische immunofenotypering zijn zeer waardevol in de zorg voor kinderen met een immunodeficiëntie.

DANKWOORD

Dit proefschrift is in de afgelopen zeven jaar tot stand gekomen naast een drukke klinische baan op diverse plaatsen: Rotterdam, Leiden en 's-Hertogenbosch. Het kan niet anders dan dat vele mensen daaraan op enigerlei wijze hun bijdrage hebben geleverd. Het is onmogelijk een ieder daar persoonlijk voor te bedanken, een aantal van hen wil ik er echter in het bijzonder uitlichten. Zonder hen zou dit proefschrift er nooit gekomen zijn.

Allereerst uiteraard professor Dr. J.J.M. van Dongen, mijn promotor. Beste Jacques, zonder jouw enthousiasme, jouw onvoorwaardelijke steun in moeilijke tijden, jouw bezielende leiding en ongezouten kritiek was ik nooit daar gekomen waar ik nu ben. Toen ik voor het eerst bij je kwam met mijn plannen heb je je ernstige twijfels uitgesproken: een dokter die in het lab wil komen werken? Aan een onderzoek dat nog vanaf de allereerste gedachte moet worden opgezet? Die moet zich eerst maar eens komen bewijzen! Jouw grote verdienste is dat áls je eenmaal met iemand afspraken maakt je je daar ook aan houdt, en je een grote steun en stimulator betoont. Ik ben je veel dank verschuldigd en hoop de samenwerking met jou - nu vanuit Den Bosch - nog lang te kunnen voortzetten.

Zonder de steun van professor Dr. R. de Groot was mijn onderzoek al in een vroege fase - na de afwijzing van mijn eerste subsidieaanvraag - blijven steken. Beste Ronald, jij hebt je altijd voor mijn plannen ingezet, al is dat de laatste jaren wat meer op afstand geworden. Voor jouw steun wil ik je bij deze ook hartelijk bedanken.

Mijn eerste schreden op het pad van de wetenschap zette ik in 1988 onder leiding van Dr. M.J.D. van Tol. Beste Maarten, ik ben je zeer erkentelijk voor mijn eerste lessen in de immunologie en het laboratoriumwerk, maar ook voor de inmiddels jarenlange samenwerking die wij verder hebben uitgebouwd in de jaren dat ik als staflid op de afdeling IHOBA in Leiden werkzaam was. Inmiddels hebben wij de samenwerking al weer uitgebreid naar de "Bosche" populatie, en ik hoop dat wij dat nog lang zo zullen doen.

Professor Dr. J.M.J.J. Vossen, medepromotor, beste Jaak, wij hebben moeilijke tijden meegemaakt. Ik ben blij dat wij nu een goede werkrelatie hebben gevonden. Ik dank je voor de mogelijkheden die je bood om mijn werk aan de immunodeficiënte Leidse patiënten ook na mijn vertrek naar Den Bosch af te maken, zodat dat werk onderdeel van dit proefschrift kon zijn.

Professor Dr. H.J. Neijens, beste Herman, mijn dank voor je altijd belangstellende houding, en voor het doorwerken van dit manuscript in korte tijd. Professor Dr. R. Benner dank ik voor zijn bereidwilligheid zitting te nemen in de

"kleine" promotiecommissie, en in korte tijd een flinke stapel manuscripten voor mij door te werken.

Marieke Comans-Bitter en Sandra de Bruin-Versteeg waren de analisten die mij steunden op het Rotterdamse immunologisch laboratorium. Marieke, ik ben je alleen al dankbaar dat je mij hebt "geschiktbevonden" voor het doen van laboratoriumwerk, bovendien heb ik ontzettend veel van je geleerd. Sandra, zonder jou was de enorme bulk aan immunofenotyperingswerk voor de longitudinale studie bij gezonde kinderen nooit afgekomen. Jij was mijn paranimf, niet alleen op de dag zelf!

Analisten van het immunologisch laboratorium in Leiden, ik ben dankbaar voor mijn eerste lessen over immunologisch laboratoriumwerk die ik van jullie kreeg. Renée Langlois van den Bergh en Jacqueline Waaijer, jullie wil ik ook bedanken voor al het immunofenotyperingswerk van de kinderen na beenmergtransplantatie, en de rompslomp die dat met zich meebracht.

Dr. F.K. Lotgering, gynaecoloog, en Dr. G.J.M. Boerma, klinisch chemicus, ik dank jullie voor jullie bereidwillige medewerking bij het verzamelen en bewerken van de monsters voor de longitudinale studie van gezonde kinderen. Datzelfde geldt voor de verpleegkundigen van de verloskamers van het Dijkzigt Ziekenhuis, en de analisten van het hematologisch laboratorium van het Sophia Kinderziekenhuis.

Dr. W.C.J. Hop, mijn dank voor de hulp bij de statistische verwerking van de resultaten uit de longitudinale studie bij gezonde kinderen. Professor Dr. P.J.J. Sauer, beste Pieter, mijn dank voor de tijd die je mij gaf om in het laboratorium bezig te zijn toen ik als staflid neonatologie bij je werkte. Dr. J. Hermans, mijn dank voor de hulp bij de statistische verwerking van de resultaten uit de studie naar het immunologisch herstel na beenmergtransplantatie. Dr. M. de Haas, beste Masja, mijn dank voor de vruchtbare samenwerking rondom het B73.1/CD16 werk. J.G. Noordzij, beste Jeroen, ik hoop dat we nog verder weten te komen met het werk rond het meisje dat geen XLA heeft!

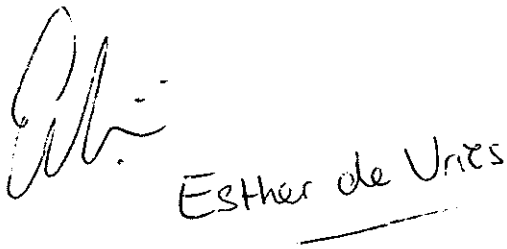
Dr. C. Weemaes, beste Corry, hartelijk dank voor je interesse en ondersteuning; ik hoop dat wij de vruchtbare samenwerking in het Bosch-Nijmeegs overleg verder zullen uit bouwen. Professor Dr. W. Kuis en dr. H. Hooijkaas, mijn dank voor het plaats nemen "achter de tafel".

Tar van Os mijn dank voor het maken van vele fraaie figuren, en Daniëlle Korpershoek voor het afwerken van diverse manuscripten.

Mijn collega's op de diverse plaatsen waar ik heb gewerkt - de Rotterdamse neonatologie, de Leidse IHOBA subafdeling, de Bosche kindergeneeskunde - ben ik dankbaar voor hun bereidwilligheid te accepteren dat ik af en toe met mijn aandacht wel erg veel bij mijn proefschrift was. Vooral bij het afronden daarvan werd de last weleens te zwaar, hetgeen mijn humeur niet altijd ten goede kwam!

Mijn ouders ben ik dankbaar voor hun stimulerende houding vanaf mijn eerste stapjes. Ik ben blij dat jullie er bij zijn om te zien waar dat allemaal toe heeft geleid. Mijn zus Laura, jij hebt vaak je steentje bijgedragen door op de kinderen te passen. Ik heb jou in je studies vaak "achter de broek gezeten" de laatste jaren - met goed resultaat! - en dat hielp mij ook weer verder. Onze goede verstandhouding mondt daarom vanzelfsprekend uit in jouw plaats als paranimf op deze "grote dag".

Arjan, jij hebt al heel wat te stellen gehad met die kinderarts-immunoloog en wetenschapper die ook nog jouw vrouw is! Hopelijk komt er nu eindelijk eens wat meer tijd. En dat geldt ook voor jullie, Sascha en Robin, die mama zat wel áltijd achter de computer de laatste tijd. Jullie vrolijke en gezellige aanwezigheid maakte het allemaal de moeite waard. En jij, Sascha, verzorgde al vele illustraties voor mij, ook de kaft van dit proefschrift, en was zelfs al eens een "leeftijdsgematchte controle". Fantastisch!



Vught, april 1999.

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INTERNATIONAL PUBLICATIONS

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2 **De Vries E**, Robben SGF, Van den Anker JN. Radiologic imaging of severe cervical spinal cord birth trauma. *Eur J Pediatr* 1995;154:230-232.

3 Van Tol MJD, Gerritsen EJA, De Lange GG, Van Leeuwen AM, Jol-van der Zijde CM, Oudeman-Gruber NJ, **De Vries E**, Radl J, Vossen JM. The origin of IgG production and homogeneous IgG components after allogeneic bone marrow transplantation. *Blood* 1996;87:818-826.

4 **De Vries E**, Koene HR, Vossen JM, Gratama JW, Von dem Borne AEGKr, Waaijer JLM, Haraldsson Á, De Haas M, Van Tol MJD. Identification of an unusual FcγReceptor IIIa (CD16) on natural killer cells in a patient with recurrent infections. *Blood* 1996;88:3022-3027.

5 De Haas M, Koene HR, Kleijer M, **De Vries E**, Simsek S, Van Tol MJD, Roos D, Von dem Borne AEGKr. A triallelic Fcγ Receptor type IIIA polymorphism influences the binding of hIgG by NK-cell FcγRIIIa. *J Immunol* 1996;156:2948-2955.

6 Levy J, Espanol-Boren T, Tomas C, Fischer A, Tovo P, Bordignoni P, Reznik I, Fasth A, Baer M, Gomez L, Sanders EAM, Tabone MD, Plantaz D, Etzioni A, Monafo V, Hammarstrom L, Abrahamsen T, Jones A, Finn A, Klemola T, **De Vries E**, Sanal O, Peitsch MC, Notarangelo LD. The clinical spectrum of X-linked hyper IgM syndrome. *J Pediatr* 1997;131:47-54.

7 Comans-Bitter WM, De Bruin-Versteeg S, Kjaervig Broe M, De Groot R, **De Vries E**, Van Dongen JJM. Intracellular CD79 expression in precursor B cells tested with the CD79 panel of monoclonal antibodies. In: T. Kishimoto, H. Kikutani, A.E.G.Kr. Von dem Borne, et al (eds), *Leukocyte typing VI, White cell differentiation antigens, Proceedings of the sixth international workshop and conference held in Kobe, Japan, 10-14 november 1996.* NewYork, 1997, pp182-184.

8 Comans-Bitter WM, De Bruin-Versteeg S, **De Vries E**, De Groot R, Van Dongen JJM. Reactivity of the B-cell blind panel antibodies on precursor B cells. In: T. Kishimoto, H. Kikutani, A.E.G.Kr. Von dem Borne, et al (eds), *Leukocyte typing VI, White cell differentiation antigens, Proceedings of the sixth international workshop and conference held in Kobe, Japan, 10-14 november 1996.* NewYork, 1997, pp238-240.

9 **De Vries E**, De Bruin-Versteeg S, Comans-Bitter WM, De Groot R, Boerma GJM, Lotgering FK, Van Dongen JJM. Longitudinal follow-up of blood lymphocyte

subpopulations from birth to 1 year of age. *J Pediatr* 1998;133:586-588.

10 De Vries E, De Bruin-Versteeg S, Comans-Bitter WM, De Groot R, Boerma GJM, Lotgering FK, Van Dongen JJM. Correction for erythroid cell contamination in microassay for immunophenotyping of neonatal lymphocytes. *Arch Dis Child Fetal Neonatal Ed* 1999, in press.

11 De Vries E, De Groot R, De Bruin-Versteeg S, Comans-Bitter WM, Van Dongen JJM. Analyzing the developing lymphocyte system of neonates and infants. *Eur J Pediatr* 1999, in press.

NATIONALE PUBLIKATIES

1 **De Vries E**, De Bruin-Versteeg S, Comans-Bitter M, Van Dongen J. Immunofenotyperen met een klein beetje bloed. *Analyse*, december 1996, pp273-276.

2 **De Vries E**. Immunologische diagnostiek stap voor stap. In: E. de Vries, C. Weemaes (eds), *19e congres Nederlandse Vereniging voor Kindergeneeskunde, "Immunologische diagnostiek in de algemene praktijk"*. Veldhoven, 1997 pp8-13.

3 **De Vries E**. Luchtweg- en KNO-infecties bij kinderen: wanneer is er meer aan de hand? *Ned Tijdschr Geneeskd Studenteneditie* 1998;I:11.

4 **De Vries E**. Infecties bij kinderen: wanneer is er meer aan de hand? *BMC Medisch Journaal* 1997;11:205-206.