

Papers

Differential expression of T cell antigens in normal peripheral blood lymphocytes: a quantitative analysis by flow cytometry

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Abstract

Aims—To obtain reference values of the level of expression of T cell antigens on normal lymphocyte subsets in order to disclose differences which could reflect their function or maturation stages, or both.

Methods—Peripheral blood from 15 healthy donors was processed by flow cytometry with triple colour analysis. For each sample phycoerythrin (PE) conjugated CD2, CD4, CD5, CD8, and CD56 monoclonal antibodies were combined with Cy5-R-phycoerythrin (TC) conjugated CD3 and fluorescein isothiocyanate (FITC) conjugated CD7; CD2- and CD7-PE were also combined with CD3-TC and CD4-FITC. Standard microbeads with different capacities to bind mouse immunoglobulins were used to convert the mean fluorescence intensity (MFI) values of the lymphocyte subsets identified by multiparametric flow cytometry into the number of antigen molecules per cell, measured as antibody binding capacity (ABC).

Results—CD4+ (helper/inducer) T cells exhibit a higher CD3 antigen expression compared with CD8+ (suppressor/cytotoxic) T lymphocytes. Within the CD4+ T cells, the CD4+CD7- subset expressed a lower level of CD3 compared with CD4+CD7+ and CD8+CD7+ cells, and higher CD2 and CD5 expression than the main CD3+CD7+ subset. Major differences in antigen expression were also detected between CD3+ T cells and CD3-CD56+ natural killer (NK) cells: NK cells exhibited higher levels of CD7 and CD56 and lower levels of CD2 and CD5 than T cells. Significantly lower CD5 expression was also detected in the small CD5+ B lymphocyte subset compared with T cells.

Conclusions—Quantitative flow cytometry with triple colour analysis may be used to detect antigen modulations in disease states and to increase the accuracy of

diagnosis by comparison with findings in normal counterparts.

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Keywords: flow cytometry, T cell antigens, peripheral blood lymphocytes.

The most common use of flow cytometry is to determine the percent of “positive” or “negative” cells for each antigen in different cell populations. However, valuable information is lost when the relative intensities of the positive cells, reflecting antigenic densities, are not considered. A cell population with a well defined phenotype, positive for one antigen, might be heterogeneous with regard to its level of expression. This may reflect different functional or maturational states, or both, or identify subpopulations on the basis of the different numbers of molecules of antigen per cell. These intensity differences may be characteristic of a specific subpopulation—for example, CD8 “dim” and CD8 “bright” lymphocytes correspond to different cellular subsets with different functional properties.¹

Recent studies have suggested that an aberrant antigen density in leukaemic cells can be used as a parameter with diagnostic and prognostic significance.^{2,3} Quantitative flow cytometry can be used to distinguish between leukaemic and normal lymphoid cells.⁴ These methods are becoming widely used in the study of pathological conditions.^{5,6} Normal lymphocyte subsets can exhibit different levels of antigen expression and this has to be taken into account in order to define “aberrant” density expression in pathological states and for the detection of minimal residual disease.

To address this problem we studied the level of expression of a series of T lymphocytic markers (CD2, CD3, CD4, CD5, CD7, CD8) in different T cell subsets from 15 healthy donors, by means of triple labelling, flow cytometry quantitative methods. This technique permits the assessment of the number of antigen molecules per cell in well defined lymphoid subsets using standard beads to convert fluorescence intensity into antibody binding

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Table 1 Monoclonal antibodies used in this study

Reagent	Fluorochrome	Volume (μ l)*	Source
CD2 (Leu-5b)	PE	20	Becton Dickinson, San Jose, CA, USA
CD3 (Tri-Color)	RPE/Cy5	5	Caltage, Burlingame, CA, USA
CD4	PE	10	Sera-Lab, Crawley Down, UK
CD4	FITC	10	Sera-Lab
CD5 (Leu-1)	PE	20	Becton Dickinson
CD7	FITC	10	Sera-Lab
CD8 (Leu-2a)	PE	20	Becton Dickinson
CD56 (Leu-19)	PE	20	Becton Dickinson

PE = phycoerythrin; RPE/Cy5 = Cy5 conjugated R-PE; FITC = fluorescein isothiocyanate.

*Amount of monoclonal antibody recommended by the manufacturer.

capacity (ABC).^{7,8} Our results show that CD2, CD3, CD4, CD5, CD7, and CD8 are expressed with different intensities on the various positive lymphocyte cell subsets. This may reflect different functions or different stages in the differentiation pathway.

Methods

BLOOD SAMPLES

Peripheral blood from 15 healthy donors (seven men and eight women, mean age 33 years) was analysed in order to evaluate the differential expression of the most common T lymphocyte antigens, which define the various cell subsets, and to obtain reference values for the quantitative, three colour, flow cytometry tests. The blood samples were collected in heparin and processed immediately.

IMMUNOSTAINING

The mononuclear cells were separated by density gradient centrifugation with Lymphoprep (Nycomed, Oslo, Norway). After washing three times with RPMI 1640 at room temperature, 2×10^6 cells were used per test. Monoclonal antibodies directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) and Cy5-RPE (TC) were appropriately combined in each tube. The monoclonal antibodies used in this study are shown in table 1. The following three colour staining protocols were applied: for each sample, PE conjugated CD2, CD4, CD5, CD8, and CD56 were combined with CD3-TC and CD7-FITC. A CD4-FITC monoclonal antibody was also used combined with CD3-TC and CD2- or CD5-PE ABCs to measure the expression of CD2 and CD5 on T helper cells as well as to assess the influence of different fluorochrome conjugations on ABC measurements. To each tube 50 μ l of 2% AB serum was added followed by the appropriate amount of each monoclonal antibody. Ten microlitres of double labelled isotypic mouse immunoglobulins (MsIgG-FITC/RDI) (Coulter) and 5 μ l of MsIgG-TC (Caltag) were used as negative controls in all experiments. The

cells were vortexed and incubated for 15 minutes at room temperature, washed three times with phosphate buffered saline (PBS) azide, resuspended in 0.5 ml Isoton (Coulter) and analysed on a flow cytometer.

FLOW CYTOMETRY ANALYSIS

Flow cytometry was performed using a FACScan (Becton Dickinson) with linear amplification (1024 channels) of the forward- and side-scatter signals (FS and SS) and logarithmic amplification (4 logdecades) of the FL1, FL2 and FL3 signals. Compensation parameters for multiparametric studies were adjusted at the start of analysis. Correlated data of 10 000 events per sample were acquired with a live gate applied on lymphocytes and stored in list mode using the Lysis II software (Becton Dickinson). Analysis was performed using Lysis II and Paint-a-Gate^{plus} software (Becton Dickinson). The latter reports the mean fluorescence intensity (MFI) of the investigated antigens for each of the seven subsets of cells resulting from the three colour analysis.

DETERMINATION OF ANTIBODY BINDING CAPACITY (QUANTIFICATION)

The MFI values of the positive cells were converted into ABC or number of molecules per cell by using Quantum Simply Cellular (QSC) Microbeads Kit (Sigma, St Louis, Missouri, USA). This is a mixture of four microbead populations of uniform size, coated with goat anti-mouse antibodies, which have differing capacities to bind mouse monoclonal antibodies. Included in the mixture is a blank population of microbeads having no specific binding capacity for mouse immunoglobulins. The accompanying software regresses the binding capacities of the microbeads, stained with each fluorochrome conjugated monoclonal antibody, against their corresponding peak channels. The regression curve permits quantitative estimates of the monoclonal antibody molecules bound to the target cells. Each monoclonal antibody was added in the appropriate amount to 50 μ l QSC beads. After one hour of incubation, the mixtures were washed and analysed according to the method used for lymphocytes and using the same instrument settings. The MFI of the respective antigen analysed on the positive lymphocyte populations was converted into molecules of antibody bound to the cell membrane (ABC) or number of antigen molecules per cell.

STATISTICAL ANALYSIS

The analysis of variance (ANOVA) was used to compare the ABC values of the different

Table 2 Expression of CD3, CD7 and CD56 antigens (measured as ABC) in different lymphocyte subsets (CD3-TC/CD7-FITC/CD56-PE immunostaining protocol). Results are expressed as mean (SD)

Lymphocyte subset	Percentage		ABC $\times 10^3$		
			CD3	CD7	CD56
CD3+CD7+CD56-	62.25 (9.86)		37.63 (8.41)	26.56 (8.49)	-
CD3+CD7-CD56-	4.35 (2.57)	p < 0.001	21.69 (7.19)	-	-
CD3+CD7-CD56+	4.12 (2.82)	p < 0.005	36.04 (14.75)	34.04 (19.67)	8.23 (4.50)
CD3+CD7 CD56+	9.95 (4.16)		-	65.46 (28.01)	12.39 (3.99)

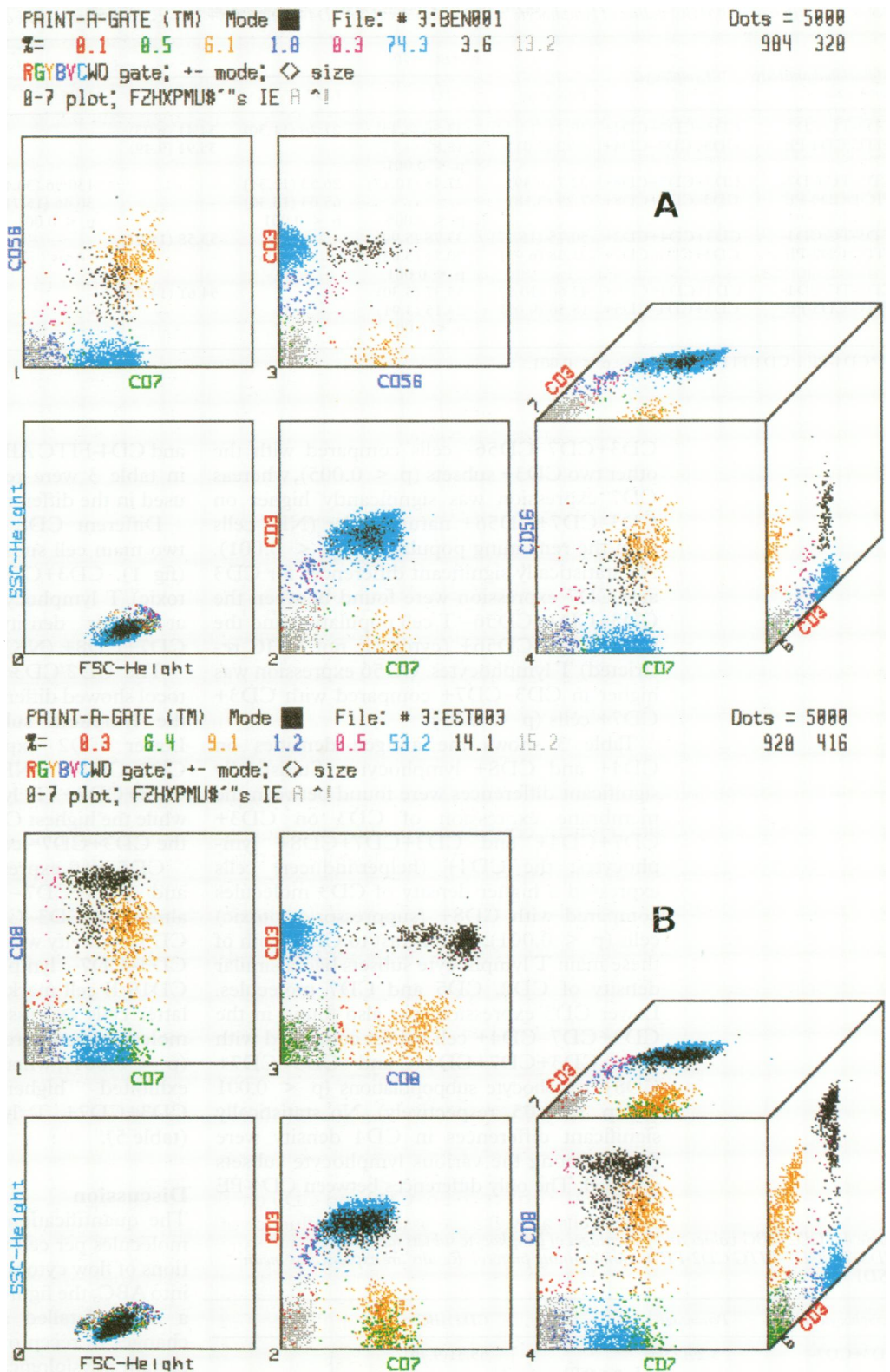


Figure 1 Flow cytometry dot plots and cube plots of lymphocytes obtained with Paint-a-Gate^{Plus} software. (A) CD3+CD7+CD56⁻ T lymphocytes are shown in cyan, CD3⁻CD7+CD56⁺ NK cells in yellow, the CD3+CD7⁻CD56⁻ cell subset in blue, and cells positive for all of the three antigens in black. In quadrant 1, the yellow subset expresses CD7 and CD56 with a higher intensity compared with the cyan and the black cells, respectively, whereas the blue subset in quadrant 2 exhibits a lower density of CD3 than the other two positive subsets (black and cyan). (B) The yellow cell subset (CD3⁻CD7+CD8⁺) in quadrant 1 shows higher CD7 and lower CD8 expression compared with the black (CD3+CD7+CD8⁺) and the cyan (CD3+CD7+CD8⁻) cells. This latter cell subset, moreover, expresses higher levels of CD3 compared with the black cells (quadrant 2).

lymphocyte subsets for each antigen; p values <0.05 were regarded as significant.

Results

The CD3/CD7/CD56 staining combination (fig 1) showed a main T cell population

CD3+CD7+CD56⁻ and three other lymphocyte subsets, CD3+CD7⁻CD56⁻, CD3+CD7+CD56⁺, and CD3⁻CD7+CD56⁺. The ABCs of CD3, CD7 and CD56 antigens on these lymphocyte subpopulations are shown in table 2. The CD3 density was lower in the

Table 3 Differences in ABC values of lymphocyte antigens expressed on CD4+ and CD8+ subsets. Results expressed as mean (SD)

Monoclonal antibody combination	Lymphocyte subset	Percentage	ABC × 10 ³					
			CD3	CD7	CD4***	CD8	CD2	CD5
CD3-TC/CD7-FITC/CD4-PE	CD3+CD7+CD4+	39.24 (14.49)	35.56 (8.43)	27.26 (11.30)	34.41 (6.03)	-	-	-
	CD3+CD7-CD4+	3.72 (2.01)	18.87 (4.75)	-	35.91 (9.49)	-	-	-
CD3-TC/CD7-FITC/CD8-PE	CD3+CD7+CD8+	22.7 (6.49)	27.48 (10.17)	26.53 (11.34)	-	130.96 (33.18)	-	-
	CD3-CD7+CD8+	7.29 (3.18)	-	65.05 (15.30)	-	30.46 (15.71)	-	-
CD3-TC/CD4-FITC/CD2-PE	CD3+CD4+CD2+	50.75 (15.17)	33.78 (5.99)	-	53.58 (15.97)	-	59.38 (9.30)	-
	CD3+CD4-CD2+	21.38 (6.95)	20.71 (3.62)	-	-	-	54.78 (12.73)	-
CD3-TC/CD4-FITC/CD5-PE	CD3+CD4+CD5+	43.60 (10.82)	33.37 (5.30)	-	54.61 (14.96)	-	-	94.33 (6.15)
	CD3+CD4-CD5+	18.50 (9.05)	23.15 (3.91)	-	-	-	-	81.85 (27.06)

***CD4-PE v CD4-FITC ABC values: p < 0.001.

CD3+CD7-CD56- cells compared with the other two CD3+ subsets (p < 0.005), whereas CD7 expression was significantly higher on CD3-CD7+CD56+ natural killer (NK) cells than the remaining populations (p < 0.001). No statistically significant differences for CD3 and CD7 expression were found between the CD3+CD7+CD56- T cell population and the CD3+CD7+CD56+ (cytotoxic not MHC restricted) T lymphocytes. CD56 expression was higher in CD3-CD7+ compared with CD3+CD7+ cells (p < 0.05).

Table 3 shows the antigen densities on CD4+ and CD8+ lymphocytes. Statistically significant differences were found between the membrane expression of CD3 on CD3+CD7+CD4+ and CD3+CD7+CD8+ lymphocytes: the CD4+ (helper/inducer) cells expressed a higher density of CD3 molecules compared with CD8+ (suppressor/cytotoxic) cells (p < 0.001). In contrast to CD3, both of these main T lymphocyte subsets had a similar density of CD2, CD5 and CD7 molecules. Lower CD3 expression was also found in the CD3+CD7-CD4+ cell subset compared with both CD3+CD7+CD4+ and CD3+CD7+CD8+ lymphocyte subpopulations (p < 0.001 and p < 0.05, respectively). No statistically significant differences in CD4 density were found among the various lymphocyte subsets analysed. The only differences between CD4-PE

and CD4-FITC ABC values (p < 0.001) shown in table 3 were related to the fluorochromes used in the different staining protocols.

Different CD8 densities characterised the two main cell subsets positive for this antigen (fig 1). CD3+CD7+CD8+ (suppressor/cytotoxic) T lymphocytes expressed CD8 antigen at higher density compared with CD3-CD7+CD8+ (NK) cells (p < 0.001).

The CD2/CD3/CD7 immunostaining protocol showed different CD2 expression in all of the lymphoid subsets identified (table 4). Lower CD2 expression was detected in CD3-CD7+ (NK) cells compared with CD3+CD7+ T lymphocytes (p < 0.001), while the highest CD2 ABC value was found in the CD3+CD7- cell subset (p < 0.01).

CD5 was expressed on both CD3+CD7+ and CD3+CD7- T lymphocytes, whereas almost all CD3-CD7+ NK cells were CD5-; CD5 positivity was also detected on a subset of CD3-CD7- lymphoid cells, positive for the CD19 B cell marker (data not shown). These latter cells expressed significantly fewer CD5 molecules compared with CD3+CD7+ T cells (p < 0.001), whereas the CD3+CD7- subset exhibited higher CD5 density than CD3+CD7+ T lymphocytes (p < 0.005) (table 5).

Discussion

The quantification of the number of antigen molecules per cell is a goal in modern applications of flow cytometry. By translating the MFI into ABC, the figures are amplified, resulting in a more detailed and sensitive detection of changes in receptor expression determined by certain physiological states, diseases or following differentiation, especially when all working conditions and staining protocols are well standardised.⁸ In flow cytometry quantification is particularly important, to have comparable results, that conditions of analysis are rigorously standardised (cell preparation procedures, fluorochromes, monoclonal antibodies used, and their different combinations, etc.). The fluorescence intensity level of individual cell surface markers can vary depending on cell preparation and staining.⁹ Usually the ABCs obtained with FITC conjugated monoclonal antibodies are higher than those obtained with other fluorochromes; moreover, in three colour

Table 4 CD2 ABC values and percentages of lymphocyte subsets identified by CD3-TC/CD7-FITC/CD2-PE immunostaining protocol. Results are expressed as mean (SD)

Lymphocyte subset	Percentage	CD2 ABC × 10 ³
CD3+CD7-	5.29(2.94)	65.51(9.79)
CD3+CD7+	69.07(7.79)	55.89(7.94)
CD3-CD7+	12.14(5.25)	42.82(8.32)

p < 0.01 (CD3+CD7- vs CD3+CD7+)
p < 0.001 (CD3+CD7+ vs CD3-CD7+)
p < 0.001 (CD3+CD7- vs CD3-CD7+)

Table 5 CD5 ABC values and percentages of lymphocyte subsets identified by CD3-TC/CD7-FITC/CD5-PE immunostaining protocol. Results expressed as mean (SD)

Lymphocyte subset	Percentage	CD5 ABC × 10 ³
CD3+CD7+	62.66(7.43)	85.78(12.33)
CD3+CD7-	5.19(3.74)	108.5 (32.03)
CD3-CD7-	1.15(6)	19.09(11.04)

p < 0.005 (CD3+CD7+ vs CD3+CD7-)
p < 0.001 (CD3+CD7- vs CD3-CD7-)
p < 0.001 (CD3+CD7+ vs CD3-CD7-)

analysis the staining intensity of certain antigens, such as CD3, is lower than with single or double staining, using the same fluorochrome.¹⁰ This is confirmed by the evidence of different CD4 ABCs obtained in this study using different FITC or PE conjugated monoclonal antibodies in two different staining protocols, as well as the discrepancies in the ABC values of some antigens obtained with different monoclonal antibody combinations reported by others.^{8 10}

In this study we describe the differences in the expression of a variety of T cell antigens on different lymphocyte subsets. CD3 antigen is part of the T cell receptor (TCR) complex and is involved in transducing stimulatory signals after antigen specific recognition.¹¹ It is highly expressed in mature T cells and is down regulated after antigen recognition and activation.¹² In agreement with Lenkei *et al*⁸ and Islam *et al*,¹⁰ we found higher CD3 expression in CD4+ (helper/inducer) compared with CD8+ (suppressor/cytotoxic) T lymphocytes. These T cell subsets require different types of antigen presentation: CD4 and CD8 are accessory molecules in the recognition of foreign antigens by T cells in association with MHC class II and I antigens, respectively.¹³ Thus, the constitutively different expression of CD3 between these main T lymphocyte subpopulations probably reflects their different mechanisms of antigen recognition and signal transduction.

Significant differences were also found in CD7 expression between T lymphocytes and NK cells. CD7 is a glycoprotein expressed early during T cell ontogeny, which is lost only during the terminal stages of T cell development^{14 15}; high CD7 expression characterises early stages of T cell differentiation, whereas its expression is lower in memory than in naive cells.¹⁶ CD7 is also positive on CD3-CD56+ NK cells. We showed significantly higher expression of CD7 on NK cells compared with CD3+ T lymphocytes. The CD7 molecule is directly involved in the NK cell activation process and in the regulation of expression and function of adhesion molecules, thus playing an important role in anchorage and other activities of NK cells.^{17 18}

The lower CD8 expression in NK cells compared with suppressor/cytotoxic CD3+ lymphocytes probably reflects the different recognition pathways of these two cell populations. CD8 is essential in the cytotoxic function of CD3+ lymphocytes¹³; in contrast NK cells have a different recognition and cytotoxicity pattern, in which other molecules are mainly involved.¹⁹

The CD56 NK marker is an adhesion molecule expressed by all lymphocytes mediating non-MHC restricted cytotoxicity.²⁰ Expression of CD56 on a subset of CD3+CD7+ T lymphocytes has been described.²¹ They are large granular lymphocytes coexpressing $\alpha\beta$ TCR, CD5, and CD8 antigens. Functionally they can exert, like NK cells, a non-MHC restricted cytotoxic activity. However, their lower level of CD56 expression compared with NK cells, as well as their CD3 and CD7 expression, comparable with that of

CD3+CD7+CD56- cells, suggests that this subset is closer to T lymphocytes than NK cells.

CD2 is also expressed both on T and NK lymphocytes, where it exerts a physiological role in adhesion and early activation processes.²² However, its density on NK cells is lower compared with mature T lymphocytes.

The ontogeny of NK cells is not yet fully elucidated. NK cells share some features with immature thymocytes.^{17 23 24} As shown in the present study, CD7 is highly expressed on NK cells whereas they exhibit low levels of CD2 and CD8. In addition, it was recently demonstrated that NK cells, like T cell progenitors, express intracytoplasmic CD3 and ζ transcripts.^{25 26} These findings suggest that T and NK cells may share a common developmental pathway within the earliest stages of T cell commitment.²⁶

The lack of CD7 in CD3+ peripheral blood lymphocytes identifies a subset of helper cells, circa 5%, which are mainly CD4+, often express activation markers and exhibit a CD45RO+ CD45RA- memory phenotype.^{15 27 28} The lower CD3 expression on this cell subset compared with the CD3+CD7+ population probably indicates that CD7- T cells represent a physiologically distinct T cell lineage or alternatively reflects a more mature postactivation state.¹⁶ This is supported further by the CD2 and CD5 overexpression shown by CD3+CD7- cells.

CD5 is expressed on all T cells, whereas it is not found on NK cells. The CD5 receptor is important in T cell proliferation and T cell activation via CD5 requires expression of the TCR.^{17 29} This T cell antigen is also expressed by a subset of B cells which probably exert an immunoregulatory role; these CD5+ B cells produce autoreactive antibodies and are involved in the pathogenesis of certain autoimmune diseases and neoplasia.²⁹ On these cells we showed, as expected on the basis of earlier studies,⁵ lower CD5 expression compared with CD3+CD7+ peripheral blood T lymphocytes. The low CD5 positivity in the B cell lineage probably identifies an early B cell maturational stage.

Quantitative flow cytometry may be of value both in clinical and biological studies. Moreover, three colour flow cytometry permits the accurate identification of different lymphocyte subsets among the main lymphoid populations, and significant information on their immunophenotypic characteristics can be obtained. The study of differential expression of antigens on the various cell subsets permits a better understanding of the nature of these subsets, their physiological role and function, the detection of alterations in pathological states, and the elucidation of the cellular origin of some T cell leukaemias.

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