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Activation antigen expression on human T cells. I. Analysis by two-colour flow cytometry of umbilical cord blood, adult blood and lymphoid tissue

P. L. AMLOT, F. TAHAMI, D. CHINN & E. RAWLINGS *Department of Immunology, Royal Free Hospital School of Medicine, London, UK*

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SUMMARY

Activation antigens (actags) were detected on T cells at low levels of intensity by carefully defining negative cells with a panel of control antibodies. The mean percentage of blood T cells from healthy volunteers that expressed actags were 22% (CD25), 54% (CD26), 38% (CD38), 12% (CD54), 6% (CD69) and 21% (HLA-DR). The variability of actag expression detected by this sensitive method was determined on healthy volunteers by repeated estimation over a year. The percentage of T cells expressing CD25 and CD26 varied no more than repeated estimation of the CD4 T cell subset, whereas other actags showed greater variability. The antigen density of these actags on T cells was determined in relation to CD4 antigen density, and for most actags ranged from 10% to 75% of the level of CD4 antigen density except for CD7 and HLA-DR, which could exceed that of CD4. Different degrees of actag expression characterized T cells from different blood and lymphoid tissues. CD26, CD38 and CD45RA were universally expressed in cord blood at higher antigen density than adult blood. This immature pattern was consistent with recent thymic emigration. CD25, CD45RO, CD54 and HLA-DR progressively increased from cord blood through adult blood to lymphoid tissues, consistent with antigen-driven activation, whereas CD26 and CD45RA decreased. CD69, a very early activation antigen, abruptly increased in lymphoid tissue, exceeding CD25 by two-to-three-fold and suggesting a pre-activation state that may not involve commitment to antigen-driven proliferation. CD7 and CD38 expression was higher in cord blood and lymphoid tissue than in adult blood, indicating both an antigen-independent and -dependent up-regulation.

Keywords T lymphocyte activation phenotype lymphoid tissue

INTRODUCTION

Antigen or mitogen stimulation of T cells leads to a sequential expression of membrane, cytoplasmic and nuclear molecules that regulate cellular activity [1] and are widely referred to as activation antigens (actags). *In vitro* stimulation leads to strong and easily measured actag expression. The IL-2 receptor (IL-2R) has been widely studied and methodology adequate to measure actags *in vitro* only detected small numbers of IL-2R$^+$ T cells (1–5%) in blood [2–4], whether single-, double- or triple-staining techniques were used [5–7]. Actags are more frequently detected on cells in lymphoid tissues, but even here the expression may not be remarkable. In rejecting organ transplants the IL-2R is detected on only about 10% of T cells by immunohistological techniques [8–10].

There are a number of methodological reasons for the low estimates of cells expressing weak membrane antigens: (i) binding studies using radiolabelled IL-2 favour detection of intermediate and high-affinity IL-2R (complexes of α-, β- and γ-chains) compared with the IL-2Rα chain (CD25) alone [5,11,12]; (ii) early flow cytometers, like fluorescent microscopy, were insensitive to weak antigen expression [5]; (iii) cluster analysis is the favoured method for analysing flow cytometry, but this encourages an arbitrary selection of gates to define 'positive' and 'negative' cells which is inappropriate for actags [6,13], where negative and positive populations form a continuum. Higher levels of CD25$^+$ cells have been reported using undefined gating methods, but with great variability upon sequential measurement [13–15]; (iv) examination of mixed T and non-T cell populations by single immunofluorescence or by using the same gating (Quadrant) in double immunofluorescence underestimates weak antigen expression on T cells because of higher non-specific staining on non-T cells [6,13]. Taking these methodological pitfalls into account, actags were measured on T cells obtained from cord blood (naive); adult blood (normal source of human experimental T cells); and lymphoid tissues (mature, activated). For the actags measured in this study, the time required for actag expression [1,16] following *in vitro* stimulation was: 2 h (CD69), 12 h (CD7 or CD25), 1 day (CD38), 1–2 days (CD54), 2 days (CD26) and 3–5 days (HLA-DR). The

Correspondence: P. L. Amlot, Academic Department of Clinical Immunology, Royal Free Hospital School of Medicine, Rowland Hill Street, Hampstead, London NW3 2PF, UK.

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Table 1. Study demographics

Source of	No.	Sex (M:F)	Median age, years (range)
Cord blood	18		0
Adult blood	47	22:25	32 (14–53)
CRF LN and blood	11	6:5	55 (28–66)
RH LN	25	11:14	30 (2–69)
Tonsils	25	9:16	9 (4–70)
MLB LN	28	17:11	62 (28–85)
HD LN	18	12:6	31 (16–80)

CRF, Chronic renal failure; HD, Hodgkin's disease; LN, lymph node; MLB, malignant B cell lymphoma; RH, reactive hyperplasia.

Diagnoses of RH were Sjögren's disease ($n = 1$), dermatopathic lymphadenopathy ($n = 2$), tuberculosis ($n = 3$), cat scratch disease ($n = 2$), necrotizing lymphadenitis ($n = 2$), strongyloides ($n = 1$), and unknown ($n = 14$).

aim of this study was to see whether actag expression on T cells could be measured reproducibly, and whether actag expression by T cells from human lymphoid tissues showed similar patterns of actag expression already determined by *in vitro* stimulation mainly using blood T cells.

MATERIALS AND METHODS

Control subjects and lymphoid tissues

Table 1 shows the demographics of the study. Heparinized venous blood was obtained from healthy controls (adult blood), patients before renal transplantation and placentas immediately after delivery (cord blood). Lymphocytes were obtained from biopsies of lymphoid tissue by mechanical separation.

Antibodies

Antibodies used were: control (APC5, IgG2a; Dr M. Parkhouse, London, UK); TCR$\alpha\beta$ (T10B9, IgM) and CD16 (Leu-11b, IgM)

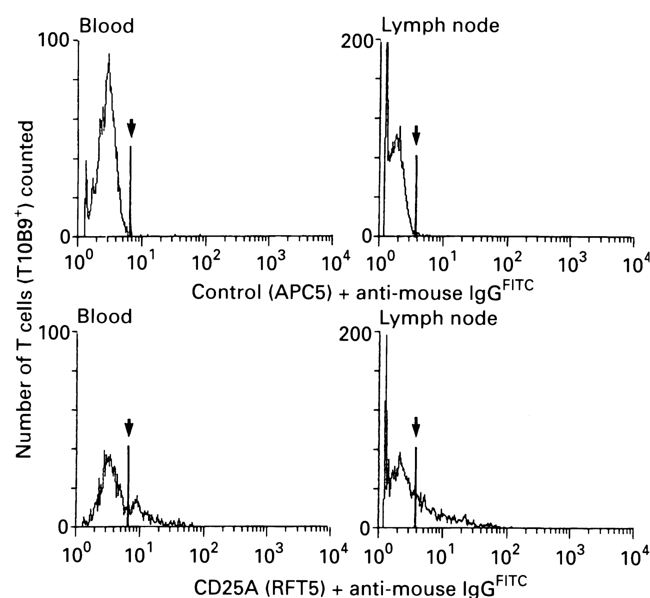

Fig. 1. FACScan histograms of TCR$\alpha\beta^+$ cells expressing CD25. Limit of the negative gate is shown by the arrowed bar.

Table 2. Different subsets expressed as a percentage (mean ± s.d.) of total lymphocytes

Group	TCR$\alpha\beta$	CD4	CD8	CD19
Cord blood	59 ± 13	42 ± 12	19 ± 9*	13 ± 5
Adult blood	67 ± 8	40 ± 7	28 ± 9	10 ± 4
CRF LN	60 ± 13	50 ± 9	11 ± 6*	30 ± 12*
RH LN	59 ± 11	44 ± 9	13 ± 5*	34 ± 11*
Tonsils	37 ± 13*	32 ± 10	5 ± 3*	55 ± 16*
MLB LN	38 ± 24*	26 ± 17*	12 ± 11*	50 ± 26*
HD LN	72 ± 11	62 ± 14*	9 ± 7*	21 ± 11

Legend as for Table 1.

*$P < 0{\cdot}01$ compared with adult blood by ANOVA and Dunnett's post-test.

TCRδ^+ T cells were rarely (<1%) detected except in adult blood (3·7 ± 3·2%), and CD16$^+$ natural killer (NK) cells were only detected in blood (cord: 20 ± 15%, and adult: 16 ± 6%).

from Professor J. Thompson (University of Kentucky); TCR$\gamma\delta$ (TCRδ1, IgG1; T Cell Sciences, Cambridge, MA); CD3 (MEM-57, IgG2a; Biogenesis, Bournemouth, UK); CD4 (RFT4, IgG1); CD7 (RFT2, IgG2a); CD8 (RFT8, IgM); CD19 (RFB9, IgG1); CD25 (RFT5, IgG2a; epitope A of CD25 [17]); CD25 (BG3, IgG1; epitope B of CD25; Dr J. Wijdenes, Diaclone, Besancon, France); CD26 (Ta1, IgG1; Coulter, Hialeah, FL); CD38 (RFT10, IgG1); CD45RA (SN130, IgG1); CD45RO (UCHL1, IgG2a; Professor P. Beverley, London, UK); CD54 (MEM-112, IgG1; Biogenesis); CD69 (TP1/55.3.1, IgG2b; Immunotech, Marseille, France); and monomorphic anti-HLA-DR, -DP and -DQ (RFDR2, IgG2a). MoAbs with the RF prefix and SN130 were all raised in the Department of Immunology, RFHSM. Normal mouse serum (NMS) was used at a 1:2000 dilution. Second layers were goat anti-mouse (GaM) IgM^{PE}, GaM IgG^{FITC}, GaM IgG1^{PE} and

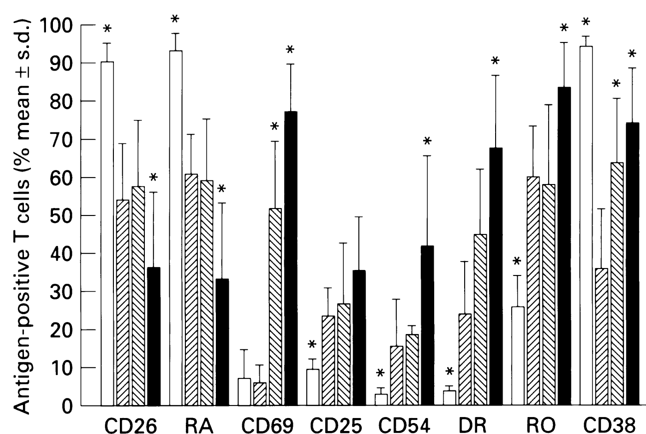

Fig. 2. Grouped actag expression on T cells from blood and lymphoid tissue. □, Cord blood; ▨, adult blood; ▧, chronic renal failure (CRF) lymph node (LN) and reactive hyperplasia (RH) LN; ■, lymphoma. Comparison with adult blood T cells by ANOVA using Dunnett's post test: $^+P < 0{\cdot}05$; $^*P < 0{\cdot}01$. CD45RA$^+$ T cells: Pearson's correlation $r = -0{\cdot}41$ CD25; 0·67 CD26; 0·14 CD38; −0·76 CD45RO; −0·36 CD54; −0·58 CD69; and −0·57 HLA-DR; $n = 178$. CD45RO$^+$ T cells: Pearson's correlation $r = 0{\cdot}57$ CD25; −0·52 CD26; −0·13 CD38; −0·76 CD45RA; 0·59 CD54; 0·61 CD69; and 0·70 HLA-DR; $n = 178$.

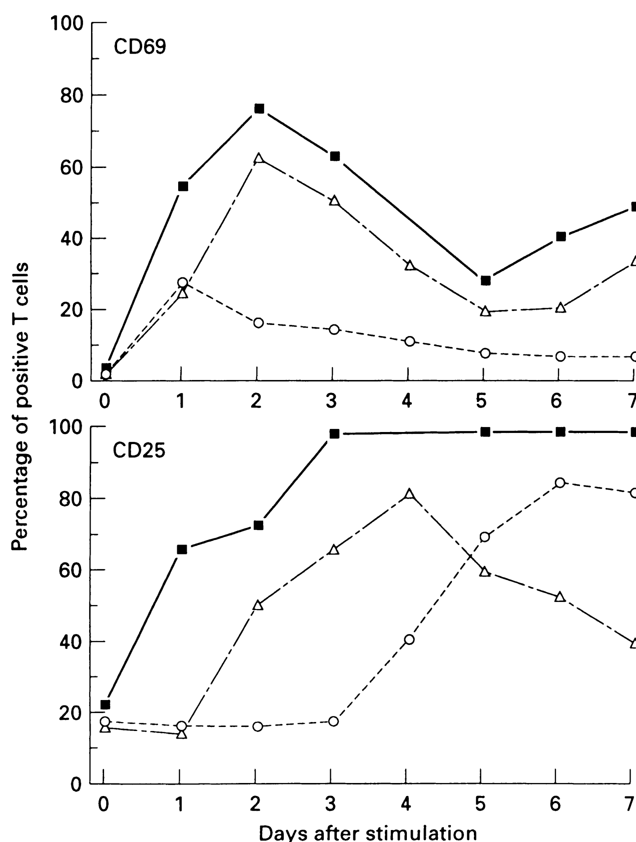

Fig. 3. Expression of actags *in vitro* after stimulation with phytohaemagglutinin (PHA), CD3 or mixed lymphocyte reaction (MLR). The figure is from a single donor and is representative of five separate experiments. ■, PHA; △, CD3; ○, MLR.

GaM IgG2a^{FITC} (Southern Biotechnology Associates, Birmingham, AL). All antibodies were titrated and used at saturating concentrations for T cells.

A directly labelled CD4 (RFT4^{FITC}) was used for quantifying CD4 on T cells using Simply Cellular beads (Flow Cytometry Standards Corp., Research Triangle Park, NC) according to the manufacturer's instructions.

Mononuclear cell separation and staining

Lymphoprep (Nycomed, Oslo, Norway)-separated mononuclear cells (MNC), with erythrocytes lysed by Hoffman's buffer, were washed and suspended in PBS containing 0·2% albumin and 0·2% azide, stained and fixed in 0·5% paraformaldehyde [18]. A FACScan (Becton Dickinson, Oxford, UK) flow cytometer and Consort 30 software were used for two-colour analysis. The FACScan was calibrated with Quantum 24 low level FITC beads at values of 1·3, 5·1, 9·8 and $17{\cdot}2 \times 10^3$ molecules of equivalent soluble fluorochrome (MESF; Flow Cytometry Standards Corp.). T cells were separated from non-T cells on the PE channel and positive from negative T cells on the fluorescent (FITC) channel. Four controls were used to set a consensus FITC gate in which 0·1–1% of T cells had to fall into the positive range with any of NMS, APC5, CD19 and TCRδ1 (Fig. 1), whereas >97% TCR$\alpha\beta$ cells fell into the positive range using CD3. Compensation settings and FITC gates established on the controls were kept constant for all other stained samples without reference to the actags.

To assess the coefficient of variation (CV), actag measurements were repeated on seven controls an average of six times over 1 year.

Standardization of actag density

MESF values were calculated directly from mean fluorescence intensity (MFI) values. Apart from MESF calibration, antigen density was standardized internally for each sample against CD4, an antigen with relatively constant density in blood. This allowed an estimate of the number of molecules per T cell, based on 3×10^4 molecules of CD4 per T cell ([19] and this study). Antigen

Table 3. Percentage of actag-positive T cells from different lymphoid tissues

Actags	CRF blood	Mean ± s.d. % of actag-positive T cells from CRF LN	RH LN	Tonsils	MLB LN	HD LN
CD26	56 ± 20	67 ± 17	54 ± 17	42 ± 15	41 ± 21	30 ± 17
CD45RA	61 ± 8	52 ± 13	63 ± 16	48 ± 10	36 ± 21	29 ± 19
CD69	8 ± 5	47 ± 17	54 ± 18	72 ± 10	76 ± 13	80 ± 13
CD25A	29 ± 7	ND	27 ± 16	20 ± 8	31 ± 14	42 ± 12
CD25B	26 ± 9	20 ± 14	21 ± 13	16 ± 4	30 ± 18	42 ± 16
CD45RO	67 ± 16	66 ± 20	54 ± 21	71 ± 11	84 ± 12	82 ± 11
CD54	29 ± 17	12 ± 9	21 ± 16	19 ± 11	43 ± 27	40 ± 19
DR	38 ± 20	39 ± 17	47 ± 18	55 ± 14	74 ± 18	59 ± 17
CD38	28 ± 10	50 ± 13	69 ± 16	81 ± 13	72 ± 14	79 ± 14

Legend as for Table 1.

Significant differences by ANOVA and Tukey's post-test ($P < 0{\cdot}05$): CRF LN *versus* RH LN for CD38 only but for all actags *versus* MLB LN or HD LN; MLB LN *versus* HD LN for CD25A and HLA-DR only; tonsils *versus* CRF LN or RH LN for CD26, CD38, CD69, CD45RA and CD45RO, or *versus* MLB LN or HD LN for CD25A, CD25B, CD54, DR, CD45RA or CD45RO.

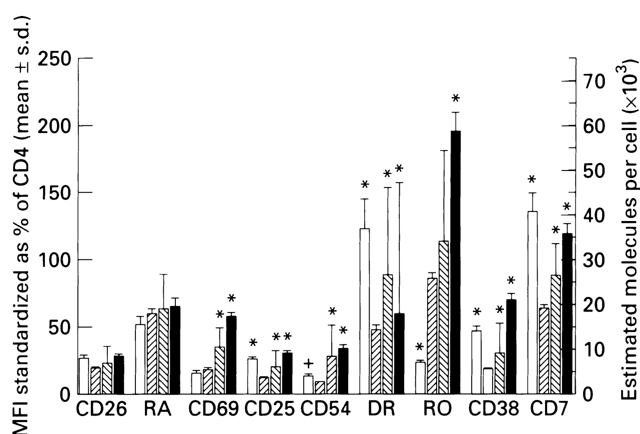

Fig. 4. Density of actag expression on T cells standardized as the percentage mean fluorescence intensity (MFI) of CD4. Legend for bars and statistics is the same as Fig. 2. Estimated CD19 expression on B cells as mean ± s.d. percentage of CD4: 61 ± 17 (cord blood), 56 ± 11 (adult blood), 52 ± 14 (chronic renal failure (CRF) lymph node (LN) and reactive hyperplasia (RH) LN), 54 ± 19 (malignant B cell lymphoma (MLB) LN and Hodgkin's disease (HD) LN) with an average of $1{\cdot}7 \times 10^4$ molecules per cell.

density was calculated as (MFI of actag/MFI of CD4) ×100% (or $\times 3 \times 10^4$ molecules per cell).

In vitro *activation*

Phytohaemagglutinin (PHA) at 1 μg/ml (Burroughs Wellcome, Beckenham, UK), mitomycin C-treated lymphoblastoid cell line (Kose) in a mixed lymphocyte reaction (MLR) and CD3 (MEM-57) at 1 μg/ml were used as stimulators. Lymphocytes were removed from culture before and after stimulation and actag expression determined on each day for a week.

Statistical analysis

The Minitab statistical program was used for analysis (Minitab Inc., State College, PA).

Table 4. Repeated measurement of T cell subsets and actags

	Mean coefficient of variation (%)	
	+ve cells	MFI*
CD45RO	9	27
CD45RA	10	33
TCRαβ	11	ND
CD25A	17	18
CD4	18	ND
CD8	20	ND
CD19	23	14
CD26	26	37
HLA-DR	39	21
CD38	52	32
CD54	58	37
CD69	83	38

*Ratio of test mean fluorescence intensity (MFI)/CD4 MFI.

RESULTS

T cells from all sources contained a two-to-six-fold excess of CD4 over CD8 (Table 2). The inappropriateness of cluster analysis to measure actags is evident from Fig. 1. For all the actags examined expression was continuous with the negative population, and distinct clusters separate from the negative population occurred infrequently. Clusters separate from negative T cells could occur with CD26 and HLA-DR in blood or lymphoid tissues or with CD69 in lymphoid tissues. CD45RA and CD45RO mostly formed distinct clusters, but double-positive cells weakly expressing the reciprocal antigen were always detected when control MoAbs were used to define the negative FITC gate.

Percentage of T cells expressing actags in blood and lymphoid tissue

Paired blood and lymph nodes (chronic renal failure (CRF)) showed more T cells expressing CD38, CD54 and CD69 in lymph nodes than blood (Table 3; $P = 0{\cdot}0003$, 0·01 and <0·0001, Student's *t*-test), but the frequency of other actags did not differ significantly. CD25A, the IL-2 binding site, was stained more frequently on T cells from blood, reactive hyperplasia (RH) LN and tonsils than CD25B, the non-IL-2 binding site [17]. However, they were stained equally on more highly activated T cells from lymphomas (Table 3) and PHA blasts (data not shown). The frequency of actag expression by CRF LN was similar to RH LN, and malignant B cell lymphoma (MLB) LN was similar to Hodgkin's disease (HD) LN, while tonsils showed an intermediate degree of activation (Table 3 and legend). For graphical comparison CRF LN and RH LN were pooled, as were MLB LN and HD LN, while tonsils were excluded for the sake of clarity. Actags separated into three groups on the basis of their staining of blood and lymphoid tissue (Fig. 2): (i) the percentage of T cells positive for CD26 and CD45RA was greatest in cord blood and least in lymphomas; (ii) the percentage of T cells positive for CD25, CD45RO, CD54, CD69 and HLA-DR was lowest in cord blood and highest in lymphomas. A high frequency of CD69 expression was typical of lymphoid tissues. The excess of CD69 over $CD25^+$ T cells found in lymphoid tissues diverged from the results of *in vitro* stimulated T cells, where disproportionate expression of CD69 was not found (Fig. 3); (iii) CD38 was high in cord blood as well as lymphoid tissue.

Correlating the degree of actag expression with CD45RA or CD45RO status, CD45RA correlated positively with CD26 but negatively with CD25, CD45RO, CD54, CD69 and HLA-DR. CD45RO showed the opposite correlation (legend to Fig. 2). The percentage of T cells with CD25, CD45RO, CD54, CD69 and HLA-DR correlated positively with each other ($r = 0{\cdot}43$–$0{\cdot}87$). $CD45RA^+RO^+$ double expression (8–10%) did not differ significantly between the groups (data not shown).

Assessment of actag density on T cells

CD26 and CD45RA antigen density remained constant despite a fall in the percentage of positive T cells from cord blood to lymphomas, while the antigen density of CD25, CD45RO, CD54, CD69 and HLA-DR increased together with the percentage of actag-positive T cells in blood and tissues (Fig. 4). CD25 and HLA-DR antigen density was greater on the infrequently positive T cells from cord blood than the more frequently positive adult blood T cells. The antigen density of CD7 and CD38 was high on cord blood T cells, lowest on adult blood and high again in tissues.

The CD4 antigen density on blood T cells was measured using Simply Cellular beads, giving a mean estimate of $3{\cdot}2 \pm 0{\cdot}2 \times 10^4$ molecules per cell (on four different individuals). An estimate of the number of actag molecules per cell was made (Fig. 4) and, when applied to CD25, showed a rise from $0{\cdot}4 \times 10^4$ to 12×10^4 on PHA-stimulated T cells by day 3 of culture. Antigens readily detected by cluster analysis had $>10^4$ molecules per cell.

Consistency of actag expression by T cells

Repeated measurements on blood from healthy individuals are summarized in Table 4. T cells expressing CD45RA and CD45RO were the most tightly regulated, with slightly greater variability for CD4, CD8 and CD25. Greatest variability was seen with CD54 or CD69 that are infrequently expressed on blood T cells.

DISCUSSION

The methodology described in this study has proved to be a sensitive measure for detecting weakly expressed antigens that merge with the negative population rather than form a separate cluster. At the same time it gave reproducible results upon repeated measurement. Use of several negative control antibodies of different IgG isotypes was important in determining the cut-off point from negative cells and overcame the variability inherent in using a single isotype-matched control MoAb. Restricting gating to T cells [20] instead of including non-T cells [6] markedly increases the sensitivity of actag detection. Confirmation of the validity of this approach was provided by patients treated with a chimaeric CD25 MoAb whose CD25 expression was inhibited while other actags were unaffected [21].

An attempt to measure actag density was made by regular calibration of the flow cytometer and a constant internal standard, CD4 [22]. The result must be regarded as semiquantitative, because the binding affinities of the MoAbs are likely to influence the result. Differences between laboratories in estimating lymphocyte subset antigen density by flow cytometry can be considerable [22–24]. Unlike previous attempts, this study uses CD4 as an internal standard. Internal standardization avoids the problems of external standards, such as cell lines with fluctuating antigen expression or fluorescent beads which cannot control for variability in daily staining procedures.

T cells from different sources were used to evaluate the methodology in the anticipation that there would be a progressive change in actag expression from cord blood through adult blood, CRF LN, RH LN and tonsils to lymphomas. This expectation was justified for both numbers and antigen density, but led to some unanticipated findings including high actag expression on tonsil T cells. CRF LN provides the nearest example of resting lymphoid tissue, but it was only marginally different from RH LN.

CD45RA and CD26 expression, markers of immature T cells

The association of CD45RA and antigen unstimulated cells has been widely reported [25,26]. CD26, a dipeptidyl peptidase IV, has on the contrary been associated with 'memory' T cells because the CD26 fraction contains responsive cells to recall antigens [27]. However, $CD26^+$, like $CD45RA^+$ T cells, show vigorous IL-2 production, mitogen responses, poor pokeweed mitogen-driven IgG synthesis, are expressed on medullary thymocytes [26–29], and in this study decrease with the rise of other actags. CD27 is another actag more highly expressed on immature T cells, and its expression parallels that of CD26 [30].

The function of actags that increase following antigen stimulation is better understood than of those associated with immature, non-primed T cells. CD25 in IL-2-driven proliferation, CD69 in pre-activation heightened responsiveness, CD54 in cell–cell adhesion, HLA-DR in antigen presentation and CD45RO in association with membrane antigens that enhance activation contrast with the poorly understood roles of CD7, CD26 and CD38.

Increasing actag expression on mature antigen-activated T cells

CD69 is the earliest membrane actag to appear following stimulation, is expressed on very few blood T cells, but undergoes a remarkable increase in lymphoid tissues, noted previously by immunohistology [31]. CD69 greatly exceeds CD25 on T cells in tissues, although the reverse is true when T cells are stimulated *in vitro* (Fig. 3). CD69 expression can be induced by ligation of CD5 or CD28 and ligands for these exist in lymphoid tissues (CD72 or CD80) capable of inducing calcium flux but not necessarily proliferation [32]. CD25 compared with CD69, HLA-DR and CD38 is relatively under-expressed in tissues and belies the findings of *in vitro* stimulation. CD25 expression precedes IL-2 binding, internalization and proliferation. Re-expression of CD25, following cell division, depends upon further antigenic or mitogenic stimulation [33,34], accounting for the strong and persistent expression of CD25 in PHA-stimulated cultures. Two influences work to restrict CD25 expression *in vivo*. Internalization of IL-2R and proliferation are dependent on CD25 density and IL-2 concentration [33], so that low concentrations of IL-2 would rapidly reverse strong CD25 expression and activated T cells expressing CD25 are susceptible to apoptosis if deprived of IL-2 [35]. Both of these influences would restrict CD25 expression *in vivo*. A conformational change in CD25 with activation is suggested by the difference in staining for CD25A and CD25B epitopes [14].

There was a steady increase in CD45RO antigen density with increasing activation (Fig. 4). Maintenance of constant CD45 expression would lead to an accumulation of CD45RO at the expense of other isoforms, CD45RA and CD45RB, supporting the concept of unidirectional shift towards the CD45RO isoform with repeated antigen-driven proliferation.

Many factors determine actag expression by T cells at different sites *in vivo*, including: the speed of up- and down-regulation following antigenic stimulation, the time T cells spend in the various body compartments, and the site, extent and type of antigenic stimulation. It could be inferred from this study that CD69 must both up- and down-regulate rapidly because of the marked difference between T cells in the blood and tissues, given a transit time between the two compartments of less than 24 h. In contrast, if the expression of CD45RO takes much longer to down-regulate than the other antigen-driven actags, then this could explain why its expression is thought to be unidirectional.

Biphasic expression of CD7 and CD38 on both immature and mature T cells

The function of CD7 is poorly understood and that of CD38 is very diverse [36]. Cord blood T cells, with high expression of CD7 and CD38, respond vigorously and rapidly to mitogens like PHA, but not to stimulation via the TCR or CD3 [37–39]. Neither of these two antigens are directly linked to antigen-driven stimulation, and both antigens are up-regulated on metabolically active cells.

Actag expression *in vivo* has revealed some interesting differences compared with *in vitro* findings. The difference in actag

expression by CD4 and CD8 T cells is addressed in an accompanying paper [40]. In quiescent lymph nodes (CRF LN) only CD38, CD54 and CD69 differed from paired blood. Studies are underway to compare more activated lymphoid tissue in parallel with blood to see if this observation remains valid.

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REFERENCES

1 Crabtree GR. Contingent genetic regulatory events in T lymphocyte activation. Science 1989; **243**:355–61.

2 Sharon M, Siegel JP, Tosato G, Yodoi J, Gerrard TL, Leonard WJ. The human interleukin 2 receptor β chain (p70). Direct identification, partial purification, and pattern of expression on peripheral blood mononuclear cells. J Exp Med 1988; **167**:1265.

3 Greene WC, Leonard WJ. The human IL2 receptor. Ann Rev Immunol 1986; **4**:69.

4 Jackson AL, Matsumoto H, Janszen M, Maino V, Blidy A, Shye S. Restricted expression of p55 interleukin 2 receptor (CD25) on normal T cells. Clin Immunol Immunopathol 1990; **54**:126.

5 Caligiuri MA, Zmuidzinas A, Manley TJ, Levine H, Smith KA, Ritz J. Functional consequences of interleukin 2 receptor expression on resting human lymphocytes. J Exp Med 1990; **171**:1509–26.

6 Ohashi Y, Takeshita T, Nagata K, Mori S, Sugamura K. Differential expression of the IL-2 receptor subunits, p55 and p75 on various populations of primary peripheral blood mononuclear cells. J Immunol 1989; **143**:3548–55.

7 Gruber R, Reiter C, Riethmuller G. Triple immunofluorescence flow cytometry, using whole blood, of $CD4^+$ and $CD8^+$ lymphocytes expressing CD45RO and CD45RA. J Immunol Methods 1993; **163**:173–9.

8 Seron D, Alexopoulos E, Raftery MJ, Hartley RB, Cameron JS. Diagnosis of rejection in renal allograft biopsies using the presence of activated and proliferating cells. Transplantation 1989; **47**:811–6.

9 Halstensen TS, Brandtzaeg P. Activated T lymphocytes in the celiac lesion: non-proliferative activation (CD25) of $CD4^+$ alpha/beta cells in the lamina propria but proliferation (Ki-67) of alpha/beta and gamma/delta cells in the epithelium. Eur J Immunol 1993; **23**:505–10.

10 Wijngaard PLJ, Tuijman WB, Meyling FHJG *et al.* Endomyocardial biopsies after heart transplantation. The presence of markers indicative of activation. Transplantation 1993; **55**:103–10.

11 Siegel JP, Sharon M, Smith PL, Leonard WJ. The IL-2 receptor β chain (p70): role in mediating signals for LAK, NK and proliferative activities. Science 1987; **238**:75–78.

12 Bich-Thuy LT, Dukovich M, Peffer NJ, Fauci AS, Kehrl JH, Greene WC. Direct activation of human resting T cells by IL2: the role of an IL2 receptor distinct from the Tac protein. J Immunol 1987; **139**:1550.

13 Reed MH, Shapiro ME, Milford EL, Carpenter CB, Kirkman RL. Interleukin 2 receptor expression on peripheral blood lymphocytes in association with renal allograft rejection. Transplantation 1989; **48**:361–6.

14 Ramos EL, Milford EL, Kirkman RL *et al.* Differential IL-2 receptor expression in renal allograft recipients treated with an anti-IL-2 receptor antibody. Transplantation 1989; **48**:415–20.

15 Kirkman RL, Shapiro ME, Carpenter CB *et al.* A randomised prospective trial of anti-Tac monoclonal antibody in human renal transplantation. Transplantation 1991; **51**:107–13.

16 Cotner T, Williams JM, Christenson L, Shapiro HM, Strom TB, Strominger JL. Simultaneous flow cytometric analysis of human T cell activation antigen expression and DNA content. J Exp Med 1983; **157**:461–72.

17 Janszen M, Buck D, Maino VC. Functional and molecular properties of CD25 monoclonal antibodies. In: Knapp W, Dorken B, Gilks WR *et al.*, eds. Leukocyte typing IV. Oxford: Oxford University Press, 1989:403–6.

18 Janossy G, Amlot PL. Immunofluorescence and immunohistochemistry. In: Klaus GGB, ed. Lymphocytes—a practical approach. Oxford: IRL Press Ltd., 1987:67–108.

19 Barclay AN, Birkeland ML, Brown MH *et al.* The leukocyte antigen facts book. London: Academic Press Ltd, 1993.

20 Ishii N, Takeshita T, Kimura Y *et al.* Expression of the IL-2 receptor γ chain on various populations in human peripheral blood. Int Immunol 1994; **6**:1273–7.

21 Amlot PL, Rawlings E, Fernando ON *et al.* Prolonged action of a chimeric IL-2 receptor (CD25) monoclonal antibody used in cadaveric renal transplantation. Transplantation 1995; **60**:748–56.

22 Poncelet P, Poinas G, Corbeau P *et al.* Surface CD4 density remains constant on lymphocytes of HIV-infected patients in the progression of disease. Res Immunol 1991; **142**:291–8.

23 Poncelet P, Carayon P. Cytofluorometric quantification of cell-surface antigens by indirect immunofluorescence using monoclonal antibodies. J Immunol Methods 1985; **85**:65–74.

24 Lenkei R, Andersson B. Determination of the antibody binding capacity of lymphocyte membrane antigens by flow cytometry in 58 blood donors. J Immunol Methods 1995; **183**:267–77.

25 Beverley PCL. Is T-cell memory maintained by crossreactive stimulation? [Review]. Immunol Today 1990; **11**:203–5.

26 Akbar AN, Salmon M, Ivory K, Taki S, Pilling D, Janossy G. Human $CD4^+$ $CD45RO^+$ and $CD4^+$ $CD45RA^+$ T cells synergize in response to alloantigens. Eur J Immunol 1991; **21**:2517–22.

27 Mattern T, Scholz W, Feller AC, Flad H-D, Ulmer AJ. Expression of CD26 (dipeptidyl peptidase IV) on resting and activated human T-lymphocytes. Scand J Immunol 1991; **33**:737–48.

28 Stein H, Schwarting R, Niedobitek G. A2 cluster report: CD26. In: Knapp W, Dorken B, Gilks WR *et al.*, eds. Leukocyte typing IV. Oxford: Oxford University Press, 1989:412–8.

29 Dang NH, Torimoto Y, Shimamura K, Tanaka T, Daley JF, Schlossman SF. 1F7 (CD26): a marker of thymic maturation involved in the differential regulation of the CD3 and CD2 pathways of human thymocyte activation. J Immunol 1991; **147**:2825–32.

30 Hintzen RQ, de Jong R, Lens MAS, van Lier RAW. CD27: marker and mediator of T-cell activation? Immunol Today 1994; **15**:307–11.

31 Schwarting R, Niedobitek G, Stein H. A4 cluster report: CD69. In: Knapp W, Dorken B, Gilks WR *et al.*, eds. Leukocyte typing IV. Oxford: Oxford University Press, 1989:428–45.

32 Vandenberghe P, Verwilghen F, Van Vaeck F, Ceuppens JL. Ligation of the CD5 or CD28 molecules on resting human T cells induces expression of the early activation antigen CD69 by a calcium- and tyrosine kinase-dependent mechanism. Immunology 1993; **78**:210–7.

33 Cantrell DA, Smith KA. Transient expression of interleukin 2 receptors. Consequences for T cell growth. J Exp Med 1983; **158**: 1895–911.

34 Depper JM, Leonard WJ, Kronke M *et al.* Regulation of interleukin 2 receptor expression: effects of phorbol diester, phospholipase C, and reexposure to lectin or antigen. J Immunol 1984; **133**:3054–61.

35 Salmon M, Pilling D, Borthwick N *et al.* The progressive differentiation of primed T cells is associated with an increasing susceptibility to apoptosis. Eur J Immunol 1994; **24**:892–9.

36 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.

37 Picker LJ, Treer JR, Ferguson-Darnell B, Collins PA, Buck D, Terstappen LWMM. Control of lymphocyte recirculation in man. I. Differential regulation of the peripheral lymph node homing receptor L-selectin on T cells during the virgin to memory transition. J Immunol 1993; **150**:1105–21.

38 Harris DT, Schumacher MJ, Locasio J *et al.* Phenotypic and functional immaturity of human umbilical cord blood T lymphocytes. Proc Natl Acad Sci USA 1992; **89**:10006–10.

39 Gerli R, Agea E, Muscat C *et al.* Activation of cord T lymphocytes III. Role of LFA-1/ICAM-1 and CD2/LFA-3 adhesion molecules in CD3-induced proliferative response. Cell Immunol 1993; **148**:32–47.

40 Amlot PL, Chinn D, Tahami F. Activation antigen expression on human T cells. II. Cytotoxic (CD8) cells. Clin Exp Immunol 1996; (submitted for publication).