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Differential staining of human $\alpha\beta$ and $\gamma\delta$ T cells by the fluorescein conjugate of an anti-CD3 monoclonal antibody

J. E. MULLERSMAN, G. WHITE* & K. S. K. TUNG *Departments of Pathology and Internal Medicine, Washington University School of Medicine, and *Barnes Hospital, St Louis, MO, USA*

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SUMMARY

The enumeration of total T cells, an important function of the clinical immunology laboratory, utilizes antibodies to CD3, the macromolecular complex associated with the antigen-specific receptors of T cells. We compared the ability of some commonly employed commercial anti-CD3 reagents to stain human peripheral blood lymphocytes. Surprisingly, the fluorescein isothiocyanate (FITC) conjugate of Coulter clone T3 (FITC–T3) stained most T cells brightly, but selectively stained $\gamma\delta$ T cells very dimly or not at all. In contrast, the other anti-CD3 reagents studied (FITC–Leu 4, PE–T3, PE–Leu 4, and indirectly labelled T3 and Leu 4) stained all T cells equivalently. Dual-colour flow cytometric analysis with FITC–T3 and PE–Leu 4 readily demonstrated a FITC–T3$^-$/PE–Leu 4$^+$ population of T cells. This unique population stained dimly or not at all with a combination of anti-CD4 and anti-CD8 monoclonal antibodies and positively with the *pan*-$\gamma\delta$ T cell antibody TCRδ1. Moreover, an excellent correlation was found between the number of FITC–T3$^-$/PE–Leu 4$^+$ cells and the number of TCRδ1$^+$ cells in 32 normal individuals. Thus, the FITC–T3$^-$/PE–Leu 4$^+$ phenotype accurately marks all $\gamma\delta$ T cells. In contrast to FITC–T3, both PE-conjugated and unconjugated T3 stained $\gamma\delta$ T cells brightly. Therefore, T3 binds to an epitope present on all T cells, but fluoresceinylation specifically attenuates this antibody's ability to bind to $\gamma\delta$ T cells. These findings indicate that the use of FITC–T3 can result in a significant and variable underestimation of peripheral blood T cell number and demonstrate further that the CD3 complexes of human $\alpha\beta$ and $\gamma\delta$ T cells are significantly different.

Keywords CD3 flow cytometry fluorescein $\gamma\delta$ T cells human

INTRODUCTION

Knowledge of the antigen-specific receptors on the surface of T cells has increased greatly during the past few years. At present, two major types of T cells, one bearing an $\alpha\beta$ heterodimeric antigen receptor and the other a $\gamma\delta$ heterodimeric antigen receptor, have been recognized and characterized in mammals (Brenner, Strominger & Krangel, 1988; Strominger, 1989). Expression of the antigen receptor of either kind of T cell requires the co-expression on the cell surface of CD3, a multimeric protein complex which is non-covalently associated with the antigen-specific receptor (Clevers *et al.*, 1988). Since the proteins of the CD3 complex are present only on the surface of T cells and not on other cells, monoclonal antibodies (MoAbs) that recognize CD3 epitopes are important *pan*-T cell markers. However, there is growing recognition that some anti-CD3 MoAbs do not stain $\alpha\beta$ and $\gamma\delta$ T cells equivalently. In fact, one MoAb, WT31, was thought until recently to bind to one of the chains of the $\alpha\beta$ T cell receptor, but it is now recognized that it also reacts with $\gamma\delta$ T cells, albeit weakly (van de Griend *et al.*, 1988), and that it is in fact an anti-CD3 MoAb (Transy *et al.*, 1989).

We have discovered that the FITC conjugate of an anti-CD3 MoAb (FITC–T3), which is widely utilized in the analysis of human lymphocytes, demonstrates much reduced staining of $\gamma\delta$ T cells as compared with $\alpha\beta$ T cells. Interestingly, this discrepancy is not shared by the antibody's PE conjugate or by the unconjugated form of the antibody.

Correspondence: Dr Kenneth Tung, Department of Pathology, Washington University School of Medicine, Box 8118, 660 S. Euclid Ave., St Louis MO 63110, USA.

MATERIALS AND METHODS

Subjects

Samples of peripheral blood were obtained from 32 healthy volunteers, aged 24–50 years, working in the Washington University Medical Center.

Antibody reagents

Mouse IgG1, Leu 2a (CD8), Leu 3a (CD4), and Leu 4 (CD3) antibodies, unlabelled or conjugated to FITC or PE, and FITC-labelled goat anti-mouse immunoglobulin and PE-streptavidin were obtained from Becton Dickinson (Mountain View, CA). The anti-CD3 MoAb Coulter clone T3 (T3) and its conjugates and biotin–goat anti-mouse immunoglobulin were procured from Coulter Immunology (Hialeah, FL). Six lots of FITC–T3 (1328A054, 1328H064, 1328H074, 1329E094, 2399F033, and 2390G053) were analysed. The δ chain-specific MoAb TCRδ1 was purchased from T Cell Sciences (Cambridge, MA).

Cell preparation and staining

Blood for flow cytometric analysis and cell counts was collected into heparin- and EDTA-containing tubes, respectively. Total and differential leucocyte counts were determined by a Coulter STAKS haematology analyser. Leucocytes for flow cytometric analysis were isolated from buffy coats by NH_4Cl-induced lysis of erythrocytes (Jackson & Warner, 1986). Subsequent manipulations of the cells prior to their fixation were carried out at 4°C. All washing steps used HBSS containing 0·1% sodium azide. The viability of the isolated leucocytes was determined by trypan blue exclusion and was always greater than 95%. For staining steps the cells were resuspended in HBSS containing 0·1% sodium azide and 1% fetal calf serum. After the labelling reagent(s) were added, the cells were incubated for 20 min and then washed twice after each labelling step. Direct staining was accomplished with commercial FITC- and PE-conjugated reagents according to the reagent manufacturers' specifications. Cells were stained indirectly using purified MoAbs and either FITC–goat anti-mouse immunoglobulin or a combination of biotin–goat anti-mouse immunoglobulin and PE–streptavidin. In dual-colour studies where both indirect and direct staining were utilized, reagents were added to the cells in a sequence that was designed to minimize reaction of the goat anti-mouse immunoglobulin conjugate with the directly labelled antibodies. The unlabelled antibody was always added to the cells first and was followed by the second antibody; the directly labelled antibodies were then added. After the final labelling step the cells were washed and then fixed with 1% paraformaldehyde.

Flow cytometry

The stained cells were studied with the aid of a FACScan flow cytometer (Becton Dickinson). The lymphocyte subpopulation was selectively gated on the basis of its characteristic side- and forward-angle light scatter. The validity of the lymphocyte gate chosen was assessed using the LeucoGate reagent (Becton Dickinson, FITC–HLe-1 and PE–Leu M3). Samples were analysed with respect to appropriate isotype- and fluorochrome-matched controls. The FACScan was calibrated daily with Calibrite beads and compensation was optimized by analysis of normal peripheral blood leucocytes, which were stored at −70°C, with FITC–Leu 3a and PE–Leu 2a.

Statistical analysis

Linear regression analysis was performed according to Deming (Cornbleet & Gochman, 1979). Discordance between estimates of $CD3^+$ lymphocyte percentage was determined using sample kurtosis as a test statistic according to Barnett & Lewis (1978).

RESULTS

FITC–T3 does not stain some T cells

When the peripheral blood leucocytes from several individuals were studied by dual-colour flow cytometric analysis using FITC–T3 and PE–Leu 4, most samples showed the expected result (Fig. 1a), with virtually all cells staining either with both reagents (T cells) or with neither reagent (non-T cells). However, a few samples contained an obvious additional population (14·0%, Fig. 1b) that stained brightly with PE–Leu 4 but negatively with FITC–T3 (FITC–$T3^-$/PE–Leu 4^+ cells).

FITC–$T3^-$ T cells are largely $CD4^-CD8^-$

Since individuals with a large number of FITC–$T3^-$/PE–Leu 4^+ cells also appeared to have a relatively large number of $CD4^-CD8^-$ T cells, we explored the discrepancy between FITC–T3 staining and PE–Leu 4 staining further. Specifically, we determined whether the FITC–$T3^-$/PE–Leu 4^+ cells were of the unusual $CD4^-CD8^-$ phenotype. First, we assessed the effect of addition of FITC–Leu 3a and FITC–Leu 2a (FITC-labelled anti-CD4 and anti-CD8 MoAbs) on the staining profiles of the FITC–$T3^-$/PE–Leu 4^+ population and the FITC–$T3^+$/PE–Leu 4^+ population. Typical results are shown in Fig. 2. In this example, the bulk (73·3%) of the lymphocytes stained brightly

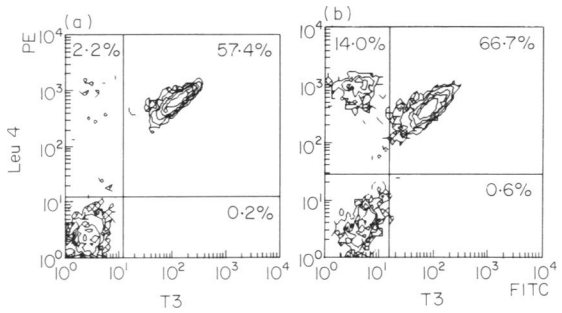

Fig. 1. Dual-colour analysis of peripheral blood lymphocytes stained with FITC–T3 and PE–Leu 4. (a) Pattern of staining observed in most normal individuals; (b) pattern of staining seen in a few normal individuals where an additional population of cells that stained brightly with PE–Leu 4 but dimly with FITC–T3 was clearly evident.

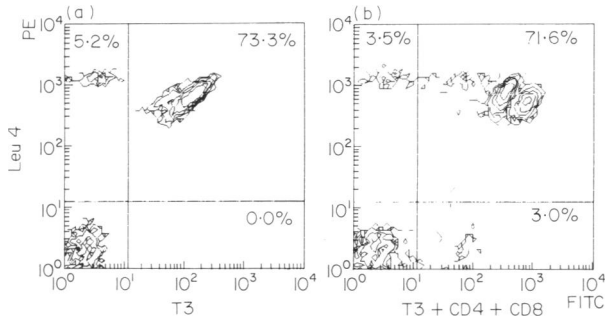

Fig. 2. Effect of addition of FITC-labelled anti-CD4 and anti-CD8 on the staining of the FITC–$T3^-$/PE–Leu 4^+ population. Seven subjects were studied; the results shown are representative. (a) Dual-colour analysis after staining with FITC–T3 and PE–Leu 4; (b) analysis as in (a), but with the addition of FITC–Leu 3a (anti-CD4) and FITC–Leu 2a (anti-CD8). The FITC–$T3^+$/PE–Leu 4^+ cells have shifted to the right and split into two distinct populations. Of the FITC–$T3^-$/PE–Leu 4^+ cells 70% are not affected, while 30% shift slightly to the right.

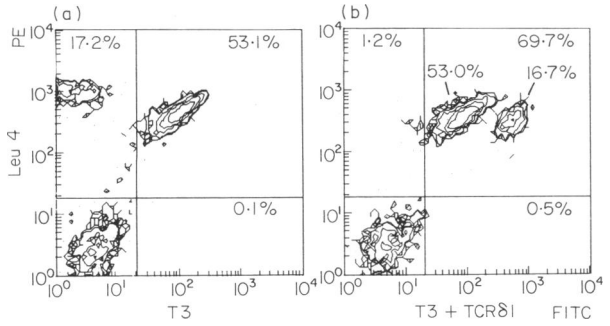

Fig. 3. Effect of addition of FITC-labelled TCRδ1 on the staining of the FITC-T3$^-$/PE-Leu 4$^+$ population. Four subjects were studied; the results shown are representative. (a) Dual-colour analysis after staining with FITC-T3 and PE-Leu 4; (b) analysis as in (a), but with the addition of TCRδ1 (indirectly labelled with FITC-goat anti-mouse immunoglobulin). Note that the CD3$^+$ cells co-stained by both FITC-T3 and PE-Leu 4 (53%) do not shift upon addition of TCRδ1. However, the FITC-T3$^-$/PE-Leu 4$^+$ cells disappear from the upper left quadrant, and a 'new' cell population (16·7%) appears to the right of the FITC-T3$^+$/PE-Leu 4$^+$ population.

with both FITC-T3 and PE-Leu 4, but a significant portion (5·2%) were FITC-T3$^-$/PE-Leu 4$^+$ (Fig. 2a). Addition of FITC-labelled anti-CD4 and anti-CD8 (Fig. 2b) yielded a marked increase in the green fluorescence of almost all of the cells in the FITC-T3$^+$/PE-Leu 4$^+$ population, indicating that they were CD4$^+$ and/or CD8$^+$. In contrast, the fluorescence of most (3·5% out of 5·2%) of the FITC-T3$^-$/PE-Leu 4$^+$ population remained unchanged, indicating that these cells were largely CD4$^-$/CD8$^-$. Seven individuals, six with no known immunological abnormality and one with rheumatoid arthritis, were examined in this way. The portion of the FITC-T3$^-$/PE-Leu 4$^+$ population that was CD4$^-$CD8$^-$ ranged from 39% to 99% (mean 66%; s.d. 19%). Additional experiments have indicated that those FITC-T3$^-$/PE-Leu 4$^+$ cells that were not CD4$^-$CD8$^-$ were largely CD8^{dim} (data not shown).

FITC-T3$^-$ T cells are γδ T cells

The demonstration that the FITC-T3$^-$/PE-Leu 4$^+$ cells were largely CD4$^-$CD8$^-$ suggested that these might be T cells with the γδ T cell receptor (Groh *et al.*, 1989). This possibility was initially supported by the determination in seven individuals that T cells that marked with the TCRδ1 reagent (γδ T cells) stained much less brightly with FITC-T3 than with FITC-Leu 4 (data not shown). Using cells from four individuals, we directly demonstrated that all of the FITC-T3$^-$/PE-Leu 4$^+$ cells were γδ T cells by evaluating the ability of TCRδ1 to change the staining profile of the FITC-T3$^-$/PE-Leu 4$^+$ population. As shown in Fig. 3, all of the FITC-T3$^-$/PE-Leu 4$^+$ cells (17%) were co-stained by TCRδ1, indicating that they were γδ T cells. In contrast, the staining profile of the FITC-T3$^+$/PE-Leu 4$^+$ cells (53%) was not affected by the addition of FITC-labelled TCRδ1, suggesting that they were αβ T cells. Finally, we found that the number of FITC-T3$^-$/PE-Leu 4$^+$ cells and the number of TCRδ1$^+$ (γδ T) cells in 32 normal individuals was strongly correlated ($r=0·96$) with the equation of the regression line ($y=0·99x-7$; x, FITC-T3$^-$/PE-Leu 4$^+$ cells/mm^3; y, TCRδ1$^+$ cells/mm^3) suggesting a one-to-one correspondence between the two. Taken together, these data indicate that all FITC-T3$^-$/PE-Leu 4$^+$ cells are γδ T cells and, conversely, that all γδ T cells are FITC-T3$^-$/PE-Leu 4$^+$. The deficient staining of γδ T cells by FITC-T3 was observed consistently with six lots of the conjugate in studies conducted over an 18-month period. The results in Table 1 demonstrate the equivalency of results obtained from the analysis of lymphocytes using three representative lots of FITC-T3.

Table 1. Pattern of staining of peripheral blood lymphocytes obtained using three representative lots of FITC-T3

	Lymphocytes staining as (%)	
FITC-T3 lot*	FITC-T3$^-$/ PE-Leu 4$^+$†	FITC-T3$^-$/ PE-Leu 4$^+$/TCRδ1$^-$‡
Subject 1 (15·1% TCRδ1$^+$)		
A	14·8	0·9
B	14·1	2·2
C	13·3	0·8
Subject 2 (10·8% TCRδ1$^+$)		
A	11·0	1·6
B	10·8	2·5
C	10·5	2·5

* Lots A, B, and C of FITC-T3 were 1329E094, 2399F033, and 2390G053, respectively.

† This cell population was delineated by dual-colour staining with FITC-T3 and PE-Leu 4 as described in Fig. 3a.

‡ This cell population was defined by dual-colour staining using TCRδ1 indirectly labelled with FITC-goat anti-mouse immunoglobulin, FITC-T3, and PE-Leu 4 as shown in Fig. 3b; the small number of cells in this population stained very dimly with PE-Leu 4 and were probably not T cells.

T3 and PE-T3 stain all T cells brightly

We also investigated whether the other forms of T3, i.e. the unlabelled MoAb and PE-T3 (marketed as RD$_1$-T3), show differential staining of αβ and γδ T cells. The ability of all three forms of T3 to stain T cells from two individuals with relatively high levels of γδ T cells in their peripheral blood (9% and 13% of lymphocytes) was studied; the corresponding forms of Leu 4 were used as controls. The percentage of lymphocytes stained with FITC-T3 was significantly different ($P<0·05$) from that stained with PE-labelled or indirectly labelled T3 or any of the Leu 4 reagents (Table 2). The difference between the number of lymphocytes detected by FITC-T3 and that detected by the other anti-CD3 reagents corresponded closely to the number of γδ T cells.

DISCUSSION

In comparing the anti-CD3 MoAbs T3 and Leu 4, we have observed that the peripheral blood lymphocytes of many normal individuals contain a small but readily detectable population of T cells that stain dimly with FITC-T3. This population is readily detected in dual-colour analyses as FITC-T3$^-$/PE-Leu 4$^+$. These FITC-T3$^-$/PE-Leu 4$^+$ cells are γδ T cells, being reactive with the γδ T cell-specific TCRδ1 MoAb. Significantly, the poor staining of γδ T cells by the FITC conjugate of T3 is not shared by either PE-conjugated or

Table 2. Staining of peripheral blood lymphocytes with different forms of T3 and Leu 4

MoAb	Lymphocytes staining with anti-CD3 MoAbs (%)	
	Subject 1	Subject 2
FITC–T3	73·9*	74·6*
PE–T3	82·7	87·0
T3/FITC–goat anti-mouse immunoglobulin	80·9	84·3
FITC–Leu 4	81·4	86·5
PE–Leu 4	81·4	87·4
Leu 4/FITC–goat anti-mouse immunoglobulin	82·6	86·3

The percentage of lymphocytes that were γδ T cells was 9% and 13% for subjects 1 and 2, respectively.

* Discordant ($P < 0·05$).

unconjugated T3, both of which stained αβ and γδ T cells equivalently.

In the recent past, several cell surface antigens, including CD2, CD3, CD5, and CD7, were all regarded as T cell-specific markers. However, CD2, CD5, and CD7 are now known to be present also on B or natural killer (NK) cells (Gadol & Ault, 1986; Lanier & Phillips, 1986; Palker *et al.*, 1986). Currently, the T cell receptor complex best distinguishes T cells from other cells both functionally and antigenically. This complex is composed of the antigen/MHC-binding chains and the relatively invariant CD3 complex (Clevers *et al.*, 1988). Since the antigen/MHC-binding chains occur as two major types (αβ and γδ) anti-CD3 MoAbs are considered to be the best reagents for identifying all mature T cells.

The most significant finding of this work is that FITC–T3, a popular marker for total T cells, does not react well with CD3 of all human T cells. In as much as FITC–T3 specifically fails to react with γδ T cells, the use of this reagent is expected to yield an underestimate of the number of total T cells. Moreover, the extent of underestimation would vary, since the percentage of γδ T cells is known to vary greatly (0–30%) among normal individuals (Groh *et al.*, 1989). Therefore, it is not appropriate to quantitate total T cells with the FITC–T3 reagent.

The failure of FITC-T3 to label γδ T cells adequately has precedent among anti-CD3 MoAbs. There is biochemical and serological evidence that the CD3 complexes of αβ and γδ T cells are not identical. The CD3δ chain of γδ T cells displays a more acidic pI than that of αβ T cells. This difference can be eliminated by treatment with glycosidases and, thus, has been interpreted as resulting from a distinct pattern of glycosylation (Krangel *et al.*, 1987). Also, there are two well-documented examples of anti-CD3 MoAbs that yield much brighter staining of αβ T cells than of γδ T cells. MoAb WT31 was thought initially to bind to a framework determinant on the antigen/MHC-binding chains of αβ T cells (Spits *et al.*, 1985). It played a key role in the discovery of γδ T cells, which stained as $OKT3^+WT31^-$ or $Leu\,4^+WT31^-$ (i.e. thought to represent $CD3^+\alpha\beta^-$; Brenner *et al.*, 1986; Lanier & Weiss, 1986). Subsequent work has revealed that WT31 does stain γδ T cells, although dimly (van de Griend *et al.*, 1988), and that it binds fibroblasts transfected with the CD3ε gene (Transy *et al.*, 1989). Therefore, WT31 is in fact an anti-CD3 MoAb. The brightness of staining of γδ cells with WT31 is enhanced by increasing the concentration of the MoAb or by pretreating cells with neuraminidase (van de Griend *et al.*, 1988). Another anti-CD3 MoAb, F101.01, has also been found to stain γδ T cells far less brightly than αβ cells (Geisler *et al.*, 1988b).

The aberrant behavior of FITC–T3 was unexpected in the light of previous reports which demonstrated that unconjugated T3 stained γδ T cells as well as it stained αβ T cells (Geisler, Larsen & Plesner, 1988a; Morio *et al.*, 1989). To the best of our knowledge this is the first report of deficient anti-CD3 staining which is limited to a particular fluorochrome-conjugated form of a MoAb. This effect is clearly dependent upon both some unique property imparted to the T3 MoAb by fluoresceinylation and some difference between the T cell receptor/CD3 complexes of αβ and γδ T cells.

The increasing importance of γδ T cells in clinical and research settings (Carbonari *et al.*, 1990; Morio *et al.*, 1990; Gouttefangeas, Bensussan & Boumsell, 1990) makes their accurate quantification a more pressing issue. Although the flow cytometric determination of T cell number has been routinely conducted in laboratories worldwide for many years, many reagents (e.g. anti-CD3 MoAbs and their conjugates) were developed before the discovery of γδ T cells, and thus were not initially tested for their ability to stain this minor T cell population. Several factors may conspire to make it difficult to detect in other anti-CD3 conjugates behavior of the sort that we are reporting here for FITC–T3. First, γδ T cells represent only a small percentage of the peripheral blood lymphocytes in most individuals. Therefore, comparison of one reagent with another on independently stained samples typically yields small differences that may not be readily discriminated from random error. Second, a more direct approach using dual-colour staining studies may not always be possible because of steric interactions between MoAbs that bind to different aspects of the T cell receptor/CD3 complex. We suggest the following approach to the evaluation of γδ T cell staining by anti-CD3 reagents: (i) identify several normal individuals in whom more than 10% of the peripheral blood lymphocytes are γδ T cells (e.g. $TCR\delta1^+$); and (ii) compare the estimate of T cell number obtained with the unknown anti-CD3 reagent to that obtained using a well-characterized reagent that stains all T cells (e.g. FITC–Leu 4). Deficient staining of γδ T cells should be apparent as a discrepancy in the two determinations which is the same size as the γδ T cell population.

Our findings are significant in the light of the important role that anti-CD3 MoAbs and their conjugates play in clinical and research settings where the quantification of T cells is required. As further work reveals the function of γδ T cells and their role in disease processes, their accurate quantification using γδ-specific and *pan*-T cell reagents will become even more important. Using the guidelines which we have proposed, it should be possible to certify the reliability of any anti-CD3 reagent with respect to the detection of γδ T cells.

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