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A false expression of CD8 antigens on $CD4^+$ T cells in a routine flow cytometry analysis

Karolina Bukowska-Straková, Jarosław Baran, Małgorzata Gawlicka and Danuta Kowalczyk

Department of Clinical Immunology, Polish-American Institute of Pediatrics, Jagiellonian University Medical College, Kraków, Poland

Abstract: The two-colour flow cytometry method applied in a routine enumeration of peripheral blood T lymphocyte subsets reveals that in some patients the entire population of $CD4^+$ lymphocytes seems to express CD8 determinants as well. However, expression of the CD8 antigens on the cell surface is much lower in comparison with typical $CD8^+$ cells. Moreover, in one-colour staining with an anti-CD8 antibody, cells with weak CD8 expression are not observed and only one typical population of $CD8^+$ lymphocytes is seen. Investigating this phenomenon, we showed that after washing patient cells in RPMI before CD4/CD8 staining, the $CD4^+$ T cell population did not show CD8 "co-expression". These results suggest that $CD4^+$ lymphocytes, which seem to co-express CD8 antigen, in fact do not have this antigen on their surface. Moreover, after the addition of patient plasma to healthy donor cells prior to CD4/CD8 staining, a weak CD8 expression on normal $CD4^+$ cells was noticed. Therefore we can assume that the agent(s) causing this phenomenon is/are present in the plasma of some patients. Altogether, these observations suggest that this phenomenon is nonspecific and probably results from cross-linking of anti-CD8 mAbs with anti-CD4 mAbs caused by factor(s) present in plasma of some patient. However, identification of that/these factor(s) requires further research.

Key words: Lymphocyte subsets - Cross-linking - CD4/CD8 staining - Flow cytometry

Introduction

Enumeration of the total number of T cells and major T cell subsets ($CD4^+$ and $CD8^+$ cells) in peripheral blood is a basic test for the diagnosis of both primary and secondary immunodeficiencies [6]. A CD4/CD8 ratio and absolute number of $CD4^+$ T cells are crucial information in monitoring AIDS progression [14].

The reference method for T cell subset enumeration is flow cytometry. This method allows for simultaneous detection of several different antigens on the cell surface [4]. However, there are some biological factors contributing to unspecific antibody binding which have a significant effect on the cytometry readout of cell phenotype, *e.g.* Fc receptors, drugs (sulfamethazine showed interaction with IgG [10]), some bacterial proteins (staphylococcal protein A, streptococcal protein G and L [7, 8, 13]) and parasitic proteins (*Toxoplasma gondii* an immunoglobulin binding protein [16]). These factors may cause false positive (when *e.g.* mAbs bind Fc receptors) or false negative (*e.g.* immunoglobulin binding proteins) cell staining, which affects the readout. It may cause problems when the analysed cell subset occurs only in a low frequency or is absent in healthy individuals but present only in pathological states. This is also true for a population of double-positive $CD4^+/CD8^+$ T lymphocytes.

Developing T cells are CD4/CD8 positive and during maturation in the thymus they lose either CD4 or CD8 and become single-positive mature T cells. Thus, co-expression of both CD4 and CD8 is rarely seen on circulating T cells. Nevertheless, some mature T cells may co-express these two molecules [11, 18]. Recently, it has been reported that within the $CD4^+CD8^+$ double-positive T cell population several subpopulations of T cells can be distinguished according to the intensity of CD4 and CD8 co-expression [17]. Based on the level of CD4 and CD8 expression, the authors described four different $CD4^+/CD8^+$ double-positive T cell populations: $CD4_{dim}CD8_{bright}$, $CD4_{med}CD8_{bright}$, $CD4_{bright}CD8_{bright}$, and

Correspondence: D. Kowalczyk, Dept. Clinical Immunology, Polish-American Institute of Pediatrics, Jagiellonian University Medical College, Wielicka 265, 30-663 Kraków, Poland;
e-mail: dkowalcz@cm-uj.krakow.pl

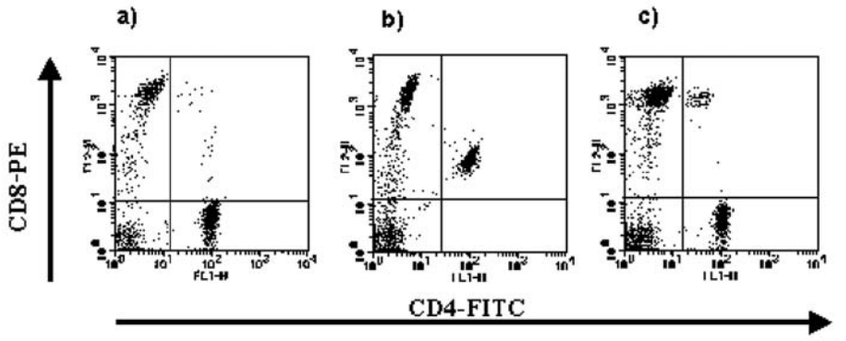

Fig. 1. FACS analysis of peripheral blood leukocytes after two-colour staining using anti-CD4 FITC (clone SK-3) and anti-CD8 PE (clone SK-1) mAbs. Dot-plots show fluorescence pattern of stained peripheral blood lymphocytes from: (**a**) healthy donor, (**b**) patient with false double-positive $CD4^+/CD8^+$ T lymphocyte population and (**c**) patient with disturbed maturation of lymphocytes in thymus.

$CD4_{bright}CD8_{dim}$. In some clinical conditions, *e.g.* thymoma, allergy or graft reception, the number of peripheral blood $CD4^+/CD8^+$ double-positive cells increases. In the case of thymoma or allergy, these lymphocytes are noticeable as additional T cell population with the expression intensity of both markers comparable to the level of CD4 and CD8 on single-positive populations, and can be described as $CD4_{bright}CD8_{bright}$ [3]. In graft recipients, part of all $CD4^+$ T cell population acquires dim CD8 expression. These cells are defined as $CD4_{bright}CD8_{dim}$ [5].

In this study we described a phenomenon of false expression of $CD8_{dim}$ antigen on $CD4_{bright}$ T cells in some patients during routine diagnostic tests of peripheral blood lymphocyte subsets. In these patients, the entire population of $CD4^+$ lymphocytes seems to express CD8 determinant as well. We suggest that it is caused by cross-linking of anti-CD8 (clone SK-1) mAbs with anti-CD4 mAbs in the presence of the factor(s) from patient plasma.

Materials and methods

Patients. The samples of peripheral blood were collected during routine diagnostic procedures from patients referred for immunological examination. Among approximately 200 patients analysed yearly, about 10% exhibited abnormal fluorescence pattern after anti-CD4 and anti-CD8 staining. These patients, both children and adults, presented different clinical characteristics; hence this effect cannot be connected to any nosological entity. No sex or age-related differences were observed.

Cells staining and flow cytometry. Peripheral blood was drawn into plastic tubes containing EDTA (Vacutainer System, BD Biosciences). 100 µl samples of whole blood were transferred to Falcon 2054 5 ml polystyrene tubes (BD Biosciences) and incubated for 30 min at 4°C with pre-titered anti-CD4 and anti-CD8 monoclonal antibodies (mAbs), labelled with FITC, PE or PE-Cy5, of the following clones:

- **anti-CD4:** SK3, SK4 (IgG_1), RPA-T4 (IgG_1) (Becton Dickinson) or MT310 (IgG_1) (DakoCytomation);
- **anti-CD8:** SK1 (IgG_1), RPA-T8 (IgG_1) (Becton Dickinson), DK25 (IgG_1) (DakoCytomation).

In some cases, cells were additionally stained with an anti-CD3 antibody conjugated with PerCP (IgG_1) (Becton Dickinson). Samples stained with appropriate isotype controls were incubated in parallel. After incubation, erythrocytes were lysed with FACS Lysing Solution (Becton Dickinson) and leukocytes were washed twice in PBS. The washed cells were suspended in 0.3 ml PBS and analysed in FASCCalibur flow cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA) using CellQuest (version 3.3) software. List mode data for 10 000 events in the lymphocyte gate were acquired.

Analysis of patient plasma influence on lymphocyte staining. 50 µl of donor blood cells obtained after cell sedimentation were mixed with 50 µl of patient plasma. Then, cells were stained with mAbs, washed and analysed as described above. In cases where double positive $CD4^+/CD8^+$ T lymphocytes were detected, cells were incubated in RPMI 1640 medium at 37°C for 30 min. After incubation samples were washed, stained and analysed by flow cytometry. Samples of blood from patients in whom we observed double-positive $CD4^+/CD8^+$ T cells were also incubated with murine IgG immunoglobulins (Sigma) or 1% newborn calf serum (Biochron, Berlin, Germany) prior to routine sample staining.

Results and discussion

Figure 1 shows dot-plots obtained in flow cytometry analysis of peripheral blood lymphocytes stained with anti-CD4 FITC and anti-CD8 PE mAbs. Figure 1a shows a normal pattern of healthy donor lymphocyte staining with the majority of $CD4^+$ cells, and a smaller population of $CD8^+$ cells, whereas figure 1b shows a shift of $CD4^+$ cells toward FL2 observed in some patients, which gives the impression that the entire $CD4^+$ subpopulation acquired CD8 antigen. This pattern of fluorescence is unusual, and differs from that observed in patients with disturbed maturation of lymphocytes in the thymus. In that case (Fig. 1c), lymphocytes stained in the same manner show typical $CD4^+$ and $CD8^+$ T cells and an additional T cell population expressing both markers at the same level as the single positive $CD4^+$ or $CD8^+$ subpopulations. These cells can be described as $CD4_{brihgt}CD8_{bright}$ T cells.

The population of $CD4^+/CD8^+$ cells observed in some of our patients also differs from the population of $CD4_{bright}CD8_{dim}$ T cells observed by Creemers *et al.* [5] in cardiac transplant recipients. In the latter patients only a part of $CD4^+$ cells (5-11%) demonstrate dim expression of CD8 antigen, whereas in our cases the entire CD4 population seems to co-express CD8.

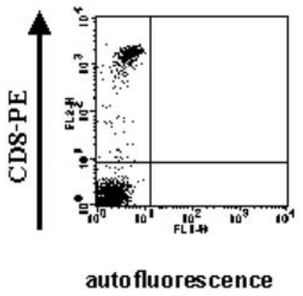

Fig. 2. Population of $CD8^+$ lymphocytes in patient with false double-positive $CD4^+/CD8^+$ T cells in a single-colour FACS analysis using anti-CD8 PE mAbs (clone SK-1).

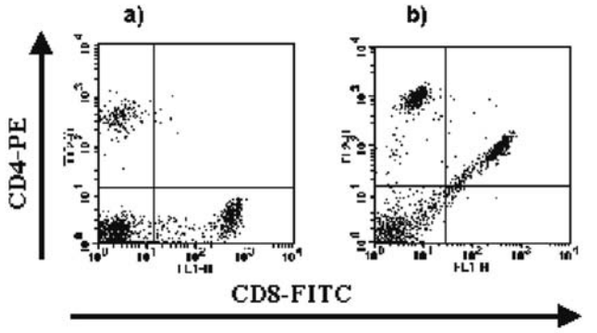

Fig. 3. FACS analysis of T lymphocyte subsets after two-colour staining using anti-CD4 PE (clone SK-3) and anti-CD8 FITC (clone SK-1) antibodies. Dot-plots show stained peripheral blood lymphocytes from (**a**) healthy donor and (**b**) patient with false double-positive $CD4^+/CD8^+$ T lymphocytes.

The question is whether the double-positive $CD4^+/CD8^+$ population observed in blood of some patients is really the result of a weak co-expression of CD8. To exclude the possibility of nonspecific binding of mAbs to dead cells, lymphocytes were stained with propidium iodide (PI). The viability of lymphocytes checked by flow cytometry exceeded 95% (data not shown). Next, we checked the co-expression of CD8 antigen on $CD4^+$ T cells. Figure 2 shows that the use of anti-CD8 PE-conjugated mAbs and single-colour flow cytometry revealed only one typical population of $CD8^+$ lymphocytes and no cells with dim expression of CD8 antigen. These data suggest that double-positive $CD4^+/CD8^+$ cells observed in two-colour staining are an artifact and their presence is apparently caused by interactions of anti-CD8 mAbs with anti-CD4 mAbs.

Interestingly, using simultaneous staining with the same clones of anti-CD4 and anti-CD8 mAbs but *vice versa* conjugated with fluorochromes (CD4-PE, CD8-FITC), "double positive" cells were also observed, but in these conditions, $CD8^+$ cells seemed to "acquire" CD4 expression. Figure 3 shows dot-plots obtained by flow cytometry analysis of lymphocytes stained with anti-CD4 PE and anti-CD8 FITC mAbs from healthy donor (Fig. 3a) in comparison to patient cells, where CD8+ cells are noticeable as a shift toward FL2 fluorescence (Fig. 3b). This finding suggests that the observed mAbs interactions result from their cross-linking. However, the cross-linking of mAbs was noticeable only as the shift of cells stained with FITC-conjugated mAbs toward FL2 channel, whereas a shift of PE stained cells towards FL1 was not seen (regardless of whether anti-CD4 or anti-CD8 mAbs were used). This can be explained by the fact that PE is a dye with high fluorescence intensity, and even a small number of PE-conjugated molecules of mAbs attached to lymphocytes may cause visible changes in the intensity of FITC-labelled cell fluorescence. On the other hand, mAbs labelled with FITC, which is a considerably dimmer fluorochrome, when attached to PE-labelled lymphocytes in a small number do not cause visible shift in fluorescence.

Another set of experiments was designed to determine whether the clones of mAbs used for cell staining are important for their cross-linking. We checked 4 different clones of anti-CD4 and anti-CD8 mAbs obtained from different vendors (Fig. 4) and found that the described phenomenon occurs only when SK1 clone of anti-CD8 mAbs was used (Fig. 1).

Subsequently, we addressed the question of whether the patient plasma is responsible for cross-linking of anti-CD4 and anti-CD8 mAbs. To exclude cytotoxic effect of patient plasma on donor cells, test with PI was applied again to check cell viability. The viability of lymphocytes checked by flow cytometry was 95% (data not shown). By incubating lymphocytes obtained from a healthy donor with patient plasma we were able to transfer the factor(s) to the cell surface and to make donor lymphocytes "double-positive" for $CD4^+/CD8^+$ in flow cytometry (Fig. 5). Therefore, we concluded that factor(s) causing cross-linking of anti-CD4 and anti-CD8 mAbs is/are present in plasma of some patients. However, after plasma removal cells remained "double-positive". Standard cell washing before incubation with mAbs did not abolish double-stained population, either. Thus, we deduced that the factor(s) is/are bound to the cell surface. The cell-attached membrane complex can be removed from the cell surface by incubating cells in RPMI 1640 medium for at least 30 minutes at 37°C (Fig. 6).

Further studies revealed that nonspecific staining can also be inhibited by incubating the patient blood with murine IgG prior to staining, or by adding 1% newborn calf serum. This would suggest a potential role for Fc receptors, especially for their soluble form [9, 15]. However, as all used mAbs were of the same isotype and the phenomenon is observed only in the presence of the SK1 clone of anti-CD8 mAbs, the role of soluble Fc receptors can be ruled out. For the same reason some bacterial products (proteins A, G, L) seem to play insignificant

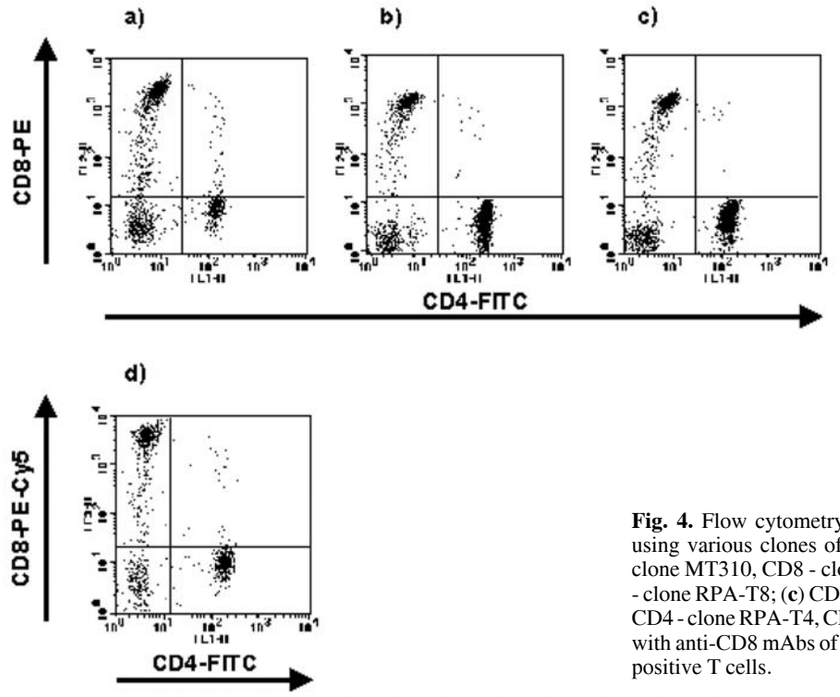

Fig. 4. Flow cytometry analysis of patient T lymphocyte subsets using various clones of anti-CD4 and anti-CD8 mAbs: (**a**) CD4 - clone MT310, CD8 - clone DK25; (**b**) CD4 - clone SK3, SK4, CD8 - clone RPA-T8; (**c**) CD4 - clone RPA-T4, CD8 - clone RPA-T8; (**d**) CD4 - clone RPA-T4, CD8 - clone RPA-T8. Staining of lymphocytes with anti-CD8 mAbs of clone other than SK1 does not show double-positive T cells.

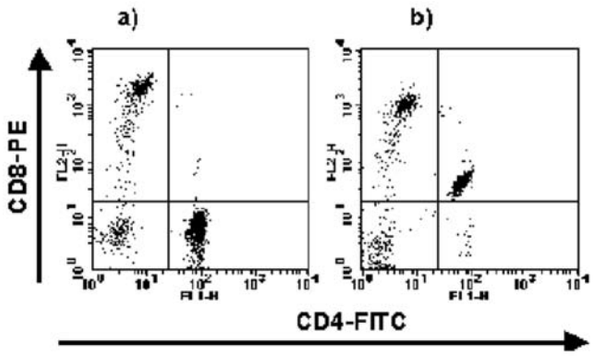

Fig. 5. FACS analysis of T cell subsets in peripheral blood samples of the same donor after (**a**) routine sample preparation and (**b**) additional sample incubation with plasma of patient with false double-positive $CD4^+/CD8^+$ T lymphocytes. The additional incubation of healthy donor lymphocytes with patient plasma makes donor lymphocytes "double-positive" for CD4/CD8.

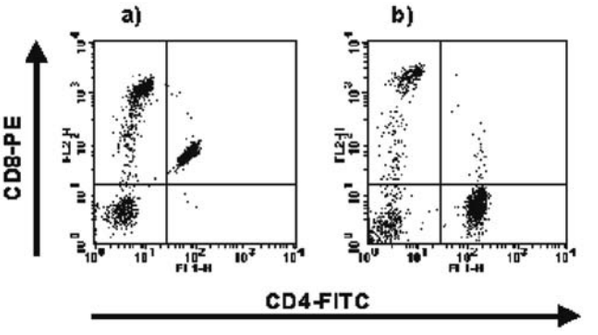

Fig. 6. FACS analysis of T cells subsets in peripheral blood of a patient with false double-positive $CD4^+/CD8^+$ T lymphocytes after (**a**) routine sample preparation and (**b**) cell incubation in RPMI 1640 medium (30 min, 37°C) and washing prior to staining. After incubation of cells in RPMI 1640 medium for at least 30 minutes at 37°C, patient lymphocytes show typical pattern of staining.

role in this phenomenon. The possible contributor involved in nonspecific binding of mAbs might be microparticles shed from $CD8^+$ lymphocytes and incorporated into the plasma membrane of $CD4^+$ T cells [1, 2, 12]. However, if this effect was mediated by microparticles in a single-color staining with anti-CD8 mAbs, an additional population with weak CD8 signal would appear. Moreover, microparticles can be ruled out as the $CD8^+$ population labelled with FITC shifts toward FL2 fluorescence what indicate that the observed phenomenon depends on cross-linking of mAbs. However, this phenomenon is undoubtedly connected with a disease process, as in some of our patients, a false expression of CD8 antigens disappeared after recovery and an anti-CD4/anti-CD8 staining returned to the normal pattern.

In summary, cross-linking of anti-CD4 and anti-CD8 mAbs is probably responsible for nonspecific staining of lymphocytes with both antibodies. This phenomenon is apparently caused by factor(s) present in plasma of some patients, and occurs only when the SK1 clone of anti-CD8 is used. To avoid nonspecific binding of this clone, cells should be incubated in RPMI 1640 medium for at least 30 minutes at 37°C. However, the identification of plasma factor(s) and the

role of the SK1 clone in this phenomenon require further studies.

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