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Severe Interference Between Retinal Angiography and Automated Four-Color Flow Cytometry Analysis of Blood Mononuclear Cells

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Abstract

Retinal angiography has become a widely used diagnostic tool. It requires the intravenous administration of the fluorescent dyes fluorescein and indocyanin green. We recently received blood taken 8 h after retinal angiography, without our knowing it. We describe the failure of an automated flow cytometry system in the enumeration of lymphocyte subpopulations in this sample. Cell enumeration was achieved by the use of the lyse-no wash MultiTEST procedure (Becton-Dickinson) together with the FACSCalibur cytometer. Absolute cell counts were obtained using TruCount beads. Data were analyzed automatically by the MultiSET and manually with the CellQuest softwares. The dot plots obtained with this sample looked quite abnormal. All monuclear cells stained brightly in the FITC channel irrespective of anti-CD3-FITC conjugate binding. This resulted in a major undercompensation for the increased spillover of the fluorescein emission into the PE-channel. PE-labeled cell and TruCount bead events coalesced. The MultiSET software failed to draw proper gatings and proved useless. Alternative manual gatings could partially rescue the analysis. Clinicians and cytometrists should be aware that, because of dye entry or binding, blood mononuclear cells collected shortly after retinal angiography are not suitable even for common cytometry applications.

Key terms

retinal angiography; flow cytometry; intravenous fluorescein; indocyanin green

Retinal angiography has become a routine diagnostic tool in ophthalmology. This technique allows us to observe microcirculation in the retina and the choroid and to evidence, among other abnormalities, capillary leakage as well as concomitant damage of surrounding cell layers. It necessitates the intravenous injection of fluorescent dyes such as fluorescein and indocyanin green. Flow cytometry on the other hand is the method of choice for the determination of lymphocyte subsets in blood, and is based on the use of monoclonal antibodies covalently labeled with various fluorochromes, including fluorescein isothiocyanate (FITC). Two publications have described interference of fluorescein administered for retinal angiography with the subsequent measurement of CD4+ T lymphocytes using simple one- or two-color flow cytometry techniques (1,2). Here we document the effects of such an interference in a widely used four-color flow cytometry system with automated computerized delineation of gates and single platform measurement of absolute cell counts. We also discuss how this phenomenon could affect other cytometric assays as well.

Materials and Methods

Lymphocyte subpopulations were measured on a two-laser (488, 635 nm) FACSCalibur Becton Dickinson (BD) flow cytometer using the lyse-no wash whole blood MultiTEST procedure and reagents. Venous blood was collected in K_3-EDTA-containing tubes. After treatment of blood with the BD FACS Lysing Solution according to

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the manufacturer's instructions, leukocytes were labeled in two separate tubes that contained a different combination of monoclonal antibodies (tube No. 1: CD3 FITC (fluorescein isothiocyanate)/CD8 PE (phycoerythrin)/CD45 PerCP (peridinin chlorophyll protein)/CD4 APC (allophycocyanin); tube No. 2: CD3 FITC/CD16 PE + CD56 PE/CD45 PerCP/CD19 APC). CD45 is present at the surface of all leukocytes, albeit at different densities characteristic of the cell type (3). CD3 and CD19 are markers of T and B lymphocytes, respectively. NK cells express CD56 and/or CD16, but not CD3. Lymphocytes were defined as $CD45^{bright}$/side angle scattered light $(SSC)^{low}$ cells and monocytes as $CD45^{medium}/SSC^{medium}$ cells (3,4). Absolute cell numbers were obtained in a single platform format by the use of titrated fluorescent beads sold in TruCount tubes (4,5). During clinical routine analyses, the gates are automatically drawn by the MultiSET software (version 1.1.2) around lymphocytes, subpopulations thereof, and TruCount beads. Calculations of the results are also performed by this software. In the present study, data acquired with MultiSET were transferred to the CellQuest software (version 3.3) for manual analysis. Calibration of the cytometer is performed daily using CaliBRITE beads. All reagents and softwares were from BD Biosciences, Allschwil, Switzerland.

Results

We report here the abnormal findings that we have obtained in the course of routine lymphocyte subsets enumeration in a blood sample from an immunodeficiency virus type1 (HIV-1)-infected patient. The patient is a 46-year-old male who acquired HIV infection by heterosexual transmission 10 years before. He is asymptomatic and does not presently receive any antiretroviral treatment. CD4+ T cell number amounted to 428 μl^{-1} and plasma viral load to 2310 copies/ml at the last examination, 3 months before. No technical problem had been encountered previously during flow cytometry analysis of his blood samples. Unexpectedly however, the current sample produced very abnormally looking dot plots, and the MultiSET software proved unable to analyze the data as the result of the lack of proper recognition and gating of the various cell populations and beads. To better figure out what was going wrong, the acquired data were transferred to the CellQuest program and analyzed by an operator. The results with tube no. 1 are presented in Figure 1, with the following features emerging (compare with the results of a reference patient on the same figure): (1) all of the patient's lymphocytes looked as if they had bound a large amount of the CD3-FITC antibody, (2) there was a massive undercompensation for the spillover of the fluorescein emission into the phycoerythrin (PE) channel, (3) the CD8-PE-labeled cells were displaced to the right of the dot plot resulting in a large overlap with the TruCount beads, which in the automated mode are delineated in the CD3/CD8 plot by the MultiSET software. Superposable abnormalities were observed with tube no. 2 from the same patient (not shown). Note that samples from other patients analyzed just before and after the case's sample did not give rise to any technical difficulty.

In an attempt to overcome the problem, a first gate was drawn around lymphocytes ($CD45^{bright}/SSC^{low}$) as usual, followed by a second gate around $CD4+/SSC^{low}$, $CD8+/SSC^{low}$, $CD19+/SSC^{low}$, and CD16+ or $CD56+/SSC^{low}$ cells. The sum of the resulting four subpopulations amounted to 103.5%, suggesting that a few cells were counted twice, possibly CD4+CD8+ T cells or $CD8^{dim}$ NK cells in the $CD8+/SSC^{low}$ gate, or CD3+CD56+ NKT cells in the NK gate. By using the number of TruCount bead events measured in the CD45/SSC plot, the absolute number of $CD4+/SSC^{low}$ lymphocytes was calculated to be 249 μl^{-1}. A similar calculation demonstrated that the absolute number of total lymphocytes obtained by cytometry (1136 μl^{-1}, $CD45^{bright}/SSC^{low}$ cells) was highly consistent with their number as obtained using a hematology analyzer (1260 μl^{-1}).

At this step, we became aware that the patient had undergone on the same day retinal angiography to investigate a rapid deterioration in the visual acuity of his right eye. To that purpose, he had received an intravenous injection of 12.5 mg of indocyanin green (sodium salt, ICG-Pulsion®, Pulsion Medical Systems, Munich, Germany), followed 4 minutes later by an intravenous injection of 400 mg of fluorescein 10% Faure® (sodium salt, Novartis Pharma, Basel, Switzerland). On the whole, ophthalmologic investigations identified either inflammatory papillitis or Leber's optic neuropathy in its acute phase as the most likely diagnosis. Blood for flow cytometry analysis was taken about 8 h after injection of the dyes. This information allowed us to understand the reason why we were facing problems with the patient's sample.

To better evidence the effect of fluorescein on our flow cytometry results, we reanalyzed the data using the two consecutive gate strategy described above, but including this time monocytes ($CD45^{medium}/SSC^{medium}$ cells) in the first gate (Fig. 2). Almost all lymphocytes and monocytes of the patient displayed a bright fluorescence in the FITC channel. The resulting undercompensation of fluorescein spillover into the PE channel is evidenced by the strong fluorescence of CD8-negative and CD16-CD56-negative cells, despite the fact that they do not bind the PE-labeled antibodies directed against those antigens. On the same PE/SSC plots, CD8+ cells are clearly recognizable as a distinct population with a still brighter fluorescence generated by the additional binding of PE-labeled antibodies. The same is true, although somewhat less evident, for CD16+ and/or CD56+ lymphocytes. As can be expected, the fluorescence in the PerCP and APC channels appears essentially unaltered.

Discussion

We illustrate here how the intravenous administration of fluorescein for retinal angiography may interfer with flow cytometry measurement of lymphocyte subsets in blood, using an up-to-date four-color system equipped with automated computerized analysis and calculation of the results. Fluorescein has been shown previously to enter all blood leukocytes (1,6). Because, at physiological pH, ~84% of fluorescein is present as a divalent anion, ~16% as a monovalent anion, and

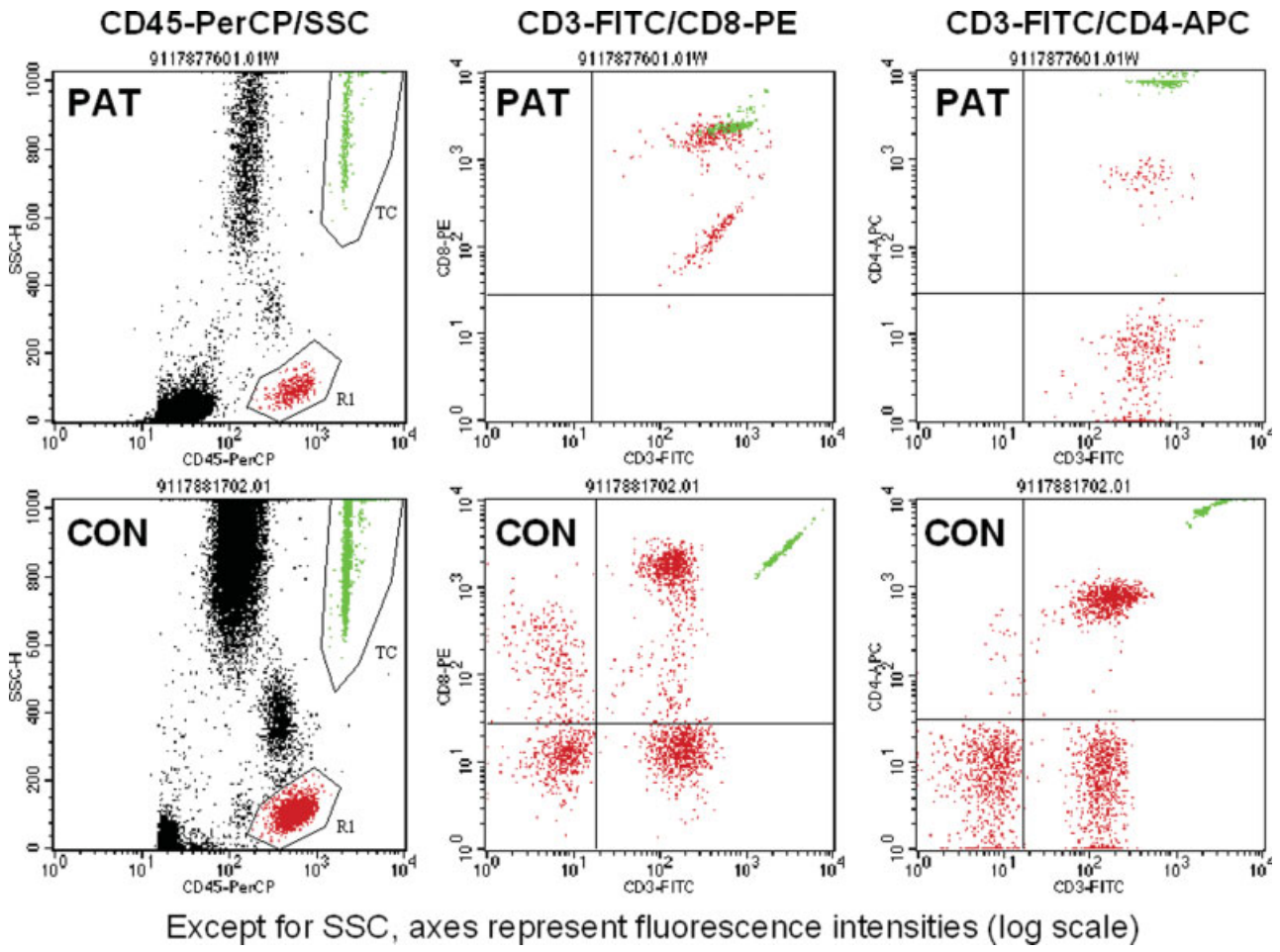

Figure 1. CellQuest analysis of T lymphocyte subsets of the case patient (PAT, upper plots) and of a reference control subject (CON, lower plots) for comparison. As shown in the left panels (CD45-PerCP/SSC), an analysis gate (R1) is drawn around lymphocytes. The case patient's sample is characterized by the apparent absence of CD3-negative cells in the CD3-FITC/CD8-PE and CD3-FITC/CD4-APC plots, as well as by the artifactual presence of two double-positive populations in the CD3/CD8 plot. The green points represent TruCount (TC) beads for absolute counts calculation. When using the automated MultiSET routine analysis software, TruCount beads are delineated in the CD3/CD8 dot plot, which proved impossible in the case of our patient.

less than 0.1% as uncharged species (7), the mechanism of entry probably involves facilitated diffusion or active transport across the plasma membrane, such as the ones described in other cell types (8,9). In the settings used here, this intracellular staining of blood cells caused a strong nonspecific fluorescent signal which did no longer allow any discrimination between non-T and T lymphocytes based on the binding of FITC-labeled anti-CD3 antibodies by the latter cells. In addition, a severe undercompensation between fluorescein/FITC and PE signals was generated, and automated absolute cell counting proved impossible because of the overlap of CD8+ T cells and TruCount beads. An alternative gating strategy bypassing CD3-FITC staining and performed manually allowed the enumeration of CD4+ T and B lymphocytes, but not of total T lymphocytes. CD8+ T cell and NK cell numbers could be obtained approximately, but would be severely overestimated in subjects with a high percentage of double-positive T cells, CD8+ NK cells, or CD56+ NKT cells. Nonspecific fluorescence in the FITC channel was also manifest for monocytes.

It is important to stress that other applications of flow cytometry targeting leukocytes are expected to be affected likewise by the intravenous administration of fluorescein, insofar as they are based on the use of FITC, PE, or other fluorochromes with similar spectral properties. These include, for example, the quantitation of expression of surface markers (10), such as CD38 on CD8+ T lymphocytes in HIV infection (11), or CD20 on B cells in chronic lymphocytic leukemia (12), immunophenotyping of malignant cells in hematology (13), intracellular cytokine production (14), and enumeration of antigen-specific cytotoxic T cells using soluble major histocompatibility class I tetramers (15). More experimental investigations, such as P-glycoprotein-mediated transport of substances out of lymphocytes (16) or measurement of CD4+ T cells proliferation by BrdU-FITC incorporation (17) are likely to be disturbed as well. Finally, in applications other than flow

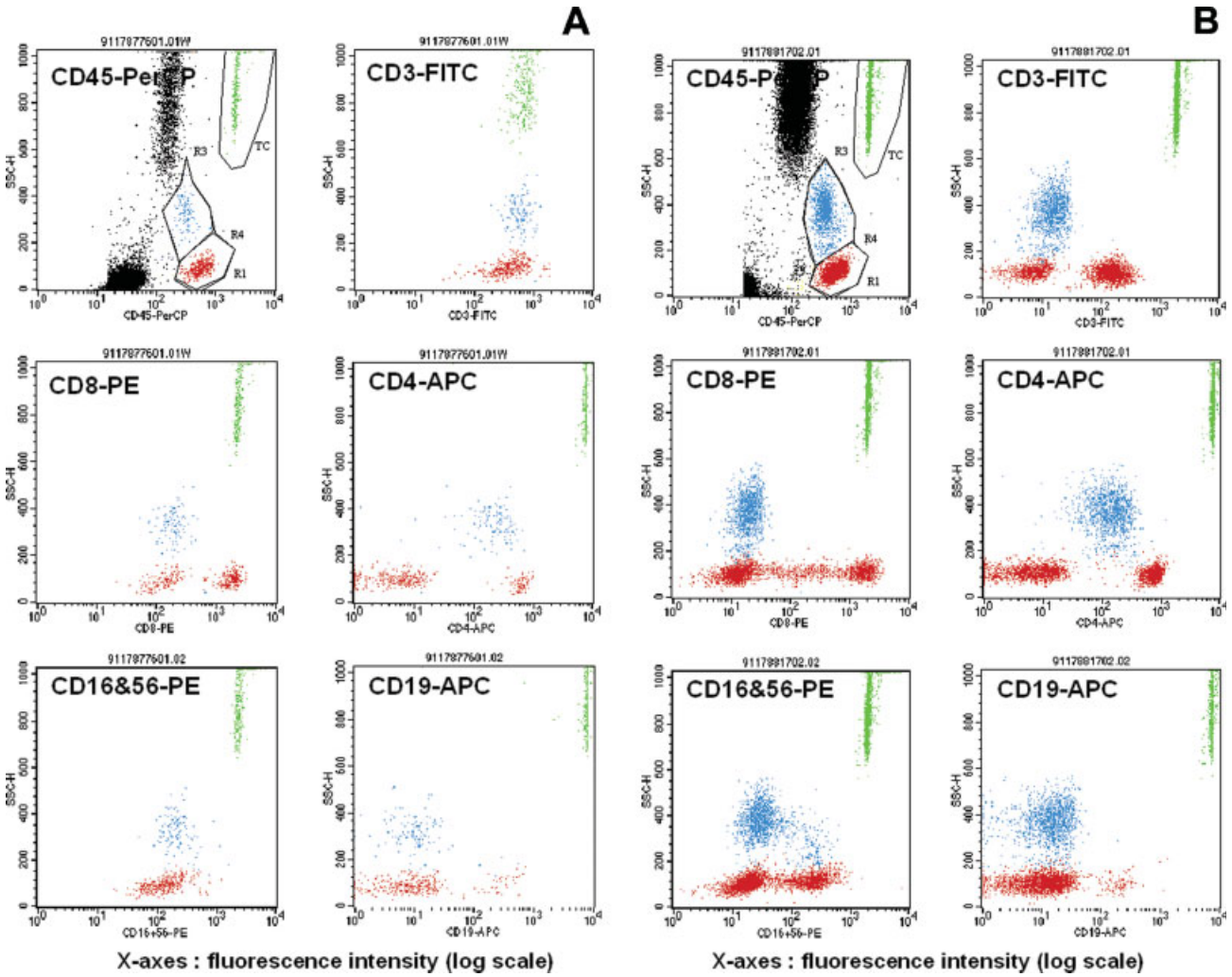

Figure 2. CellQuest analysis of single markers (*X*-axis) on lymphocytes (red) and monocytes (blue). Lymphocytes and monocytes are differentiated by the intensity of their right angle scattering of light (*Y*-axis. Low SSC, lymphocytes. Higher SSC, monocytes). As in Figure 1, the green points represent TruCount (TC) beads (highest SSC). (**A**) Case patient: Note (i) the artifactual fluorescence of monocytes, similar to that of non-T lymphocytes, in the FITC channel; (ii) the false positivity of cells that do not bind PE-labeled antibodies in the PE channel; and (iii) the absence of significant interference in the PerCP and APC channels. (**B**) Reference subject: CD8+ lymphocytes may contain doubly positive T cells or NK cells in addition to singly positive cytotoxic T cells. CD56+ lymphocytes may include NKT cells together with NK cells. Note also in the CD16 and 56-PE/SSC plot the major (CD16−) and minor (CD16+) monocyte subsets. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cytometry such as fluorescence microscopy (18), the phenomenon will certainly cause severe interference too.

In addition to fluorescein, our patient had also received the anionic dye indocyanin green (ICG), which has been reported to bind to artificial phospholipid bilayers (19), and hence presumably to cell plasma membranes. It is unlikely that ICG produced interference during standard flow cytometry analysis as performed in this study. Indeed, while the dye can be excited by the 635 nm light produced by the red diode laser in the cytometer used here, its emission peak lies in the infrared, and the small fraction of light emitted as low as 750 nm should be blocked by the bandpass filter in front of the photomultiplier tube collecting APC emission (19,20). However this situation might change if the use of infrared fluorochromes, as proposed for example by Stewart et al. for CD45 staining (21), gained widespread acceptance.

As a conclusion, fluorescein retinal angiography may strongly interfere with automated multiparameter measurements of mononuclear cell subpopulations. The outcome is expected to differ according to the combination of labeled antibodies used, especially the specificity of the FITC-labeled one. Ophthalmologists and clinicians in general should be aware that blood for cytometric applications should be taken prior to fluorescein injection or at least 24 h after (2). Cytometrists for their part should bear such an interference in mind when facing analytical difficulties such as the ones described here.

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