

Fluorescent Phycobiliprotein Conjugates for Analyses of Cells and Molecules

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ABSTRACT The synthesis of a novel class of reagents for fluorescence analyses of molecules and cells is reported. These compounds consist of a highly fluorescent phycobiliprotein conjugated to a molecule having biological specificity. Phycoerythrin-immunoglobulin, phycoerythrin-protein A, and phycoerythrin-avidin conjugates were prepared. These conjugates bind specifically to beads containing a covalently attached target molecule and render them highly fluorescent. Femtomole (10^{-15} mole) quantities of phycoerythrin conjugates can be detected because of the high extinction coefficient ($\epsilon_M = 2.4 \times 10^6 \text{ cm}^{-1} \text{ M}^{-1}$ for 2.4×10^5 daltons) and high fluorescence quantum yield ($Q = 0.8$) of the phycobiliprotein moiety. An important feature of these conjugates is that they emit in the orange-red spectral region, where background fluorescence is less than at shorter wavelengths. Phycoerythrin conjugates are well-suited for two-color flow cytofluorimetric analyses employing a single excitation line. The distributions of Leu antigens (also called OKT antigens) on the surface of T-lymphocytes were analyzed using fluoresceinated antibody as the green-fluorescent stain and biotinylated antibody counter-stained with phycoerythrin-avidin as the red one. This one-laser two-color analysis showed that cells express Leu-3a and Leu-3b or neither antigen. In contrast, the distributions of Leu-2a (a marker of suppressor and cytotoxic T-cells) and Leu-3a (a marker of helper and inducer T-cells) are mutually exclusive. These studies show that phycobiliprotein conjugates can be applied to fluorescence-activated cell sorting and analysis, fluorescence microscopy, and fluorescence immunoassay.

Single-cell analysis and sorting by flow cytofluorimetry, fluorescence microscopy, and other fluorescence techniques are important methods in cell biology (1–4). The scope and value of these experimental approaches are closely tied to the availability of suitable fluorescent probes. In particular, there is a need for orange and red-fluorescent probes to complement fluorescein, a green emitter, in analyzing for multiple markers on cells and macromolecules. Rhodamine and other currently available longer wavelength emitters are not optimal in this regard. For example, fluorescein and rhodamine cannot both be excited with high efficiency by a single laser line. It seemed to us that phycobiliproteins, a naturally occurring family of highly fluorescent macromolecules, could serve as the fluorescent moieties of a broad range of specific reagents for analyses of cells and molecules. Phycobiliproteins are constituents of the light-harvesting apparatus of blue-green bacteria, red algae, and cryptomonads (5, 6). The light energy absorbed by these proteins is funneled by dipole-dipole energy transfer to chlorophyll and then used in photosynthesis. Some characteristics of phycobiliproteins that make them well-suited for fluorescence analyses are: (a) they contain multiple bilin chromo-

phores and hence have extremely high absorbance coefficients over a wide spectral range (Table I); (b) they have high fluorescence quantum yields (~ 0.8) that are constant over a broad pH range; (c) their strong visible absorption bands start at ~ 440 nm and their intense emission bands begin at 550 nm and extend into the red (Fig. 1); (d) their fluorescence is not quenched by most biomolecules; (e) they are highly soluble in aqueous solution; and (f) they are stable in solution and in solid state and can be stored for long periods.

We report here the synthesis of conjugates of phycobiliproteins with molecules having biological specificity—namely, immunoglobulins, protein A, biotin, and avidin. These conjugates, which have the desirable fluorescence properties of the native phycobiliprotein, can be used for a variety of fluorescence analyses. Here we show that phycobiliprotein conjugates are excellent reagents for two-color fluorescence analyses of single cells using a fluorescence-activated cell sorter (FACS). The distributions of Leu antigens (also known as OKT antigens), a family of proteins on the surface of human T-lymphocytes (14–19), were analyzed using fluorescein as the green emitter and phycoerythrin as the red emitter by staining with

TABLE I
Spectroscopic Properties of Representative Phycobiliproteins

Protein*	Mol wt $\times 10^{-3}$	Absorption			Emission		References
		λ_{\max} nm	ϵ ($M^{-1} \text{ cm}^{-1}$ $\times 10^{-5}$)	$A_{1\%}^{1\text{cm}}$	λ_{\max} nm	Q	
R-Phycoerythrin	240	565	19.6	81.7	578	0.82	7, 8
B-Phycoerythrin	240	545	24.1	100.4	575	0.98	9, 10
C-Phycocyanin	36.7	620	2.81	76.5	650	0.51	11, 12
Allophycocyanin	33	650	2.32	70.3	660	0.68	10, 13

* The molecular weights given are those of the phycobiliprotein at high dilution in phosphate-buffered saline. For sources of these proteins, see the references cited.

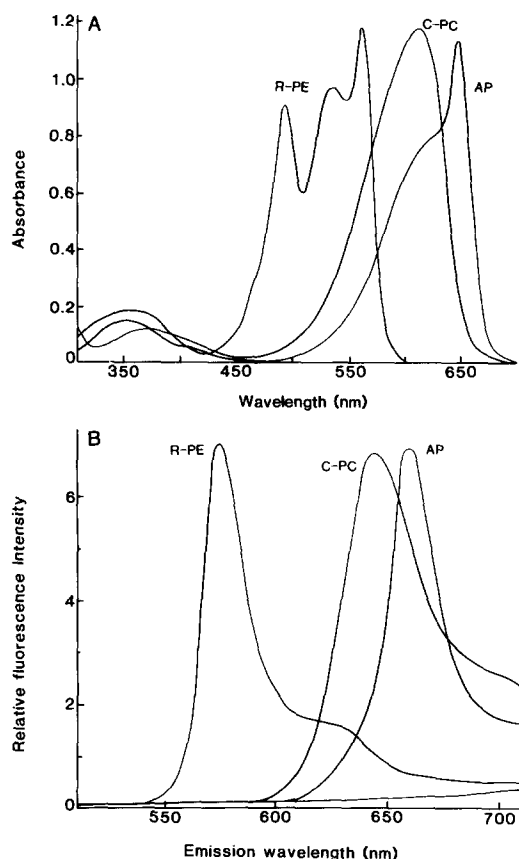


FIGURE 1 (A) Absorption and (B) fluorescence emission spectra of R-phycoerythrin (R-PE), C-phycocyanin (C-PC), and allophycocyanin (AP).

(a) a fluoresceinated antibody directed against a particular Leu antigen, and (b) a biotinylated antibody against a different Leu antigen, followed by phycoerythrin-avidin. The densities of pairs of Leu antigens on the surface of individual cells were determined by measuring the green and red fluorescence emitted by cells excited with a single laser line.

MATERIALS AND METHODS

Preparation of Phycobiliproteins

R-phycoerythrin was purified from red algae, *Gastroclonium coulteri* (Rhodymeniales), which were collected from Stillwater Cove, Monterey Peninsula, CA. The fresh algal tissue was washed with distilled water, suspended in 50 mM sodium-phosphate buffer at pH 7.0, and blended for 3 min at the highest speed setting of an Osterizer blender. The homogenate was filtered through several layers of cheese cloth and residual particulate matter removed by low speed centrifugation. The supernatant was brought to 60% of saturation with solid

$(\text{NH}_4)_2\text{SO}_4$. All of the above steps were carried out at 4°C. The precipitate was collected by centrifugation, resuspended in 60% of saturation $(\text{NH}_4)_2\text{SO}_4$ in 50 mM sodium phosphate, pH 7.0, and slurried with DEAE-cellulose (microgranular; Whatman, Inc., Chemical Separation Div., Clifton, NJ). The slurry was packed into a column. The column was developed stepwise with decreasing concentrations of $(\text{NH}_4)_2\text{SO}_4$ in 50 mM sodium phosphate, pH 7.0, down to 10% of saturation. At that point elution was completed with 200 mM sodium phosphate, pH 7.0. The phycoerythrin eluted between 10% saturation $(\text{NH}_4)_2\text{SO}_4$ -50 mM sodium phosphate, pH 7.0, and 200 mM sodium phosphate, pH 7.0. The eluate was concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation, re-dissolved in 50 mM sodium phosphate, pH 7.0, and $(\text{NH}_4)_2\text{SO}_4$ added to 10% of saturation at 4°C. The protein crystallized under these conditions upon standing at 4°C. The purification and characterization of this R-phycoerythrin will be described in detail elsewhere (D. J. Lundell and A. N. Glazer, manuscript in preparation).

Synechococcus 6301 C-phycocyanin (20), *Anabaena variabilis* allophycocyanin (21), and B-phycoerythrin (9) were prepared as described previously.

Preparation of Phycoerythrin-Avidin

A 50 μl aliquot of 1 mg/ml N-hydroxysuccinimidobiotin (Sigma Chemical Co., St. Louis, MO or Biosearch, San Rafael, CA) in dimethylsulfoxide was added to 1 ml of 2.7 mg/ml R-phycoerythrin (or B-phycoerythrin) in 50 mM sodium phosphate, pH 7.5, to give a reagent/phycoerythrin molar ratio of 13. The use of avidin and biotin in labeling studies has been described previously (22, 23). After 90 min at room temperature, the reaction was quenched by the addition of 10 μl , 100 mM glycyl-glycine, pH 7.5, and then dialyzed for 3 d at 4°C against 50 mM sodium phosphate, pH 6.8. 1 ml of this mixture of biotinylated phycoerythrin (Biot-PE) and unmodified phycoerythrin was added slowly with stirring to 1 ml of 5 mg/ml avidin in the same buffer. The molar ratio of tetrameric avidin to phycoerythrin was 20. This mixture of phycoerythrin-avidin conjugates (PE-avidin), phycoerythrin, and avidin was fractionated by high-pressure liquid chromatography.

Preparation of Phycoerythrin-Immunoglobulin G (PE-IgG)

Thiolated phycoerythrin was prepared by the addition of 600 μl of 15.5 mg/ml iminothiolane hydrochloride (Sigma Chemical Co.) (24) to 1.2 ml of 3.6 mg/ml R-phycoerythrin in 125 mM sodium phosphate, pH 6.8. After 90 min at room temperature, the reaction mixture was dialyzed overnight at 4°C against 50 mM sodium phosphate, pH 6.8, and then for 2 d against pH 7.5 buffer. Titration of an aliquot with 5,5'-dithiobis-(2-nitrobenzoic acid) showed that the average content of sulfhydryl groups per phycoerythrin molecule was 8.

A 30- μl aliquot of 1.1 mg/ml N-succinimidyl 3-(2-pyridylthio)-propionate (SPDP) (Pharmacia Fine Chemicals, Piscataway, NJ) (25) in ethanol was added to 700 μl of 4.2 mg/ml immunoglobulin G in 50 mM sodium phosphate, pH 7.5. The immunoglobulin was a monoclonal $\gamma 1$ mouse anti-allotype antibody having specificity for the α allotype of the mouse $\gamma 2a$ subclass (26). The molar ratio of SPDP to IgG was 5.3. The reaction was allowed to proceed for 2.5 h at room temperature. Thiolated phycoerythrin (400 μl of 1.7 mg/ml in the same buffer) was added to 500 μl of this reaction mixture. The molar ratio of activated IgG to thiolated phycoerythrin was 4.7. After 12 h at room temperature, 100 μl of 80 mM sodium iodoacetate was added to block any remaining sulfhydryl groups.

Preparation of Phycoerythrin-Protein A

To 0.5 ml B-phycoerythrin (4.08 mg/ml) in 0.1 M sodium phosphate-0.1 M NaCl, pH 7.4, 10 μl of SPDP in anhydrous methanol (2.65 mg SPDP/ml) was added to give an SPDP/protein molar ratio of 10. The reaction was allowed to

proceed at 22°C for 50 min and was terminated by applying the reaction mixture to a column of Sephadex G-25 (1.0 × 17 cm) equilibrated with 100 mM sodium phosphate-0.1 M NaCl, pH 7.4. The phycoerythrin peak, eluted with the same buffer, was collected and stored at 4°C.

To 0.5 ml protein A (Sigma Chemical Co.) from *Staphylococcus aureus* (27, 28), 2 mg/ml, in 100 mM sodium phosphate-100 mM NaCl, pH 7.4, 2.6 μl of the above methanolic SPDP solution was added to give an SPDP/protein molar ratio of 9.5. After 40 min at 22°C, the reaction was terminated by the addition of 25 μl of 1 M dithiothreitol in the pH 7.4 buffer. After 25 min at 22°C, the reaction mixture was subjected to gel filtration as described above and the protein A peak collected.

Appropriate volumes of the phycoerythrin-S-S-pyridyl derivative and of the thiolated protein A were mixed to give a molar ratio of phycoerythrin to protein A of 1:2. The reaction mixture contained 0.77 mg phycoerythrin/ml and 0.27 mg of protein A/ml. After 6 h at 22°C, the reaction mixture was stored at 4°C, and the phycoerythrin-protein A conjugate generated in this manner was used without further purification.

Preparation of Target Beads

IgG-Sepharose beads were prepared by adding 1.2 ml of 5.2 mg/ml IgG to 2 ml of a slurry of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals). The immunoglobulin was a mouse γ2a myeloma antibody of the α allotype (29). After 2 h of end-over-end mixing at room temperature, the reaction was quenched by the addition of 2 ml 1 M glycine, pH 7.5. The beads were then washed exhaustively. Ovalbumin-Sepharose beads and avidin-Sepharose beads were prepared similarly. Biotin-Sepharose beads were prepared by reacting ovalbumin-Sepharose beads with N-hydroxysuccinimidobiotin.

Spectroscopic Measurements

Absorption spectra were obtained on a Beckman model 25 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). Fluorescence spectra were obtained on a Perkin-Elmer model 44B fluorimeter equipped with a DCSCU-2 corrected emission spectra unit, or on a Spex Fluorolog instrument. Fluorescence microscopy was carried out using a Zeiss Universal microscope with epi-illumination optics.

High-pressure Liquid Chromatography

Coupling reactions were followed by high-pressure liquid chromatography on a Waters instrument with a Varian G3000SW gel filtration column which separates molecules primarily according to their hydrodynamic radii. For analyses, 20 μg protein was applied in a volume of 10–20 μl. In preparative experiments, 750 μl of sample was applied and 400-μl fractions were collected. The eluting buffer was 200 mM sodium phosphate, pH 6.8, and the flow rate was 1 ml/min.

Fluorescence Staining of Lymphocytes

Human peripheral blood leukocytes were prepared using Ficoll-Hypaque gradients. Viability counts of the cells recovered were done by staining the cells with acridine orange and ethidium bromide and counting fluorescent cells with a fluorescence microscope using standard fluorescein optics. This dye combination stains live cells green and dead cells orange-red (30). Cell preparations were always >95% viable.

The anti-Leu antibodies used in this study were all monoclonally derived hybridoma antibodies kindly provided by the Becton, Dickinson & Co. Monoclonal Center (Mountain View, CA). The green fluorescence signal came from directly fluoresceinated antibodies. The red fluorescence signal came from biotinylated antibodies counter-stained with phycoerythrin-avidin. Fluorescent antibody staining was done in one or two steps. Directly fluoresceinated antibodies were incubated with 10⁶ cells in 50 μl of HEPES-buffered (10 mM) RPMI-1640 medium (deficient in phenol red and biotin) containing 5% (vol/vol) horse serum and 0.02% (wt/vol) azide for 20 min on ice. The amount of antibody added had been previously determined to be optimal for staining this number of cells. For two-color staining, both the directly fluoresceinated antibody and the biotinylated antibody were incubated with 10⁶ cells in 50 μl of medium for 20 min on ice. After washing the cells twice with medium, the phycoerythrin-avidin conjugate was added to the cells in 50 μl of medium. This mixture was incubated for an additional 20 min on ice before finally washing the cells three times in medium. Cells were resuspended in 0.5 ml of medium for fluorescence analyses using the fluorescence-activated cell sorter.

Fluorescence-activated Cell Analyses

A modified Becton, Dickinson & Co. Fluorescence-activated Cell Sorter (FACS II) was used for fluorescence analyses of single cells. A 560 nm dichroic

mirror divided the emission into a shorter wavelength component (the "green" channel) and a longer wavelength component (the "red" channel). Logarithmic amplifiers with a 3.5 decade logarithmic range were used for both channels; hence our fluorescence intensity data are shown on a logarithmic rather than a linear scale. Electronic compensation (31) corrected for fluorescence spillover into the red channel and for phycoerythrin spillover into the green channel. These corrected signals will be referred to as green and red fluorescence. Our fluorescence data are displayed as contour maps which resemble topographic maps. The contour lines depict cell density on a linear scale. The number of cells in a region of the map is proportional to the volume represented by the contours in that region. The computer program that acquires and displays FACS data in this manner was written by Wayne Moore (Stanford University) and will be described in detail elsewhere.

RESULTS

Phycoerythrin can readily be coupled to other proteins. The PE-avidin conjugate was prepared by biotinylating phycoerythrin and then adding an excess of avidin. An average of one biotin per phycoerythrin was incorporated when a 13-fold molar excess of N-hydroxysuccinimidobiotin was reacted with phycoerythrin for 90 min. The reaction mixture obtained upon subsequent addition of a large excess of avidin was analyzed by high-pressure liquid chromatography (Fig. 2). Phycoerythrin-avidin conjugates eluted first, followed by phycoerythrin and biotinylated phycoerythrin and then by avidin, as expected on the basis of their hydrodynamic radii. As shown, in Fig. 2C, the reaction mixture contained a substantial amount of PE-avidin conjugates (peaks 1 and 2), in addition to phycoerythrin (peak 3) and avidin (peak 4). It seems likely that peak 2 corresponds to a conjugate containing one avidin molecule

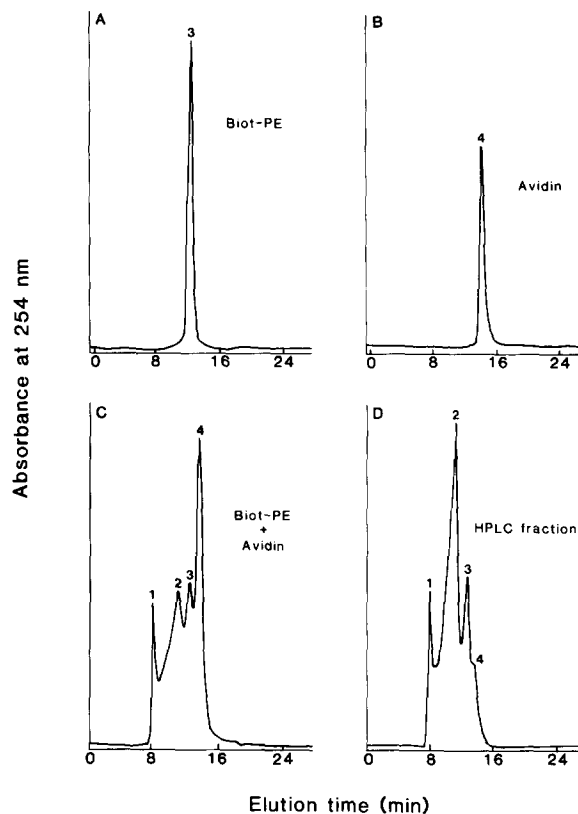


FIGURE 2 High-pressure liquid chromatography elution profiles of (A) biotinylated phycoerythrin (*Biot-PE*), (B) avidin, (C) a mixture of *Biot-PE* and avidin, and (D) fraction 2 from the chromatographic run shown in C. The labels on the peaks denote: 1, void volume fraction containing phycoerythrin-avidin conjugates; 2, included volume fraction containing phycoerythrin-avidin conjugates; 3, unmodified or biotinylated phycoerythrin; and 4, avidin.

joined to one molecule of biotinylated phycoerythrin, whereas peak 1 and the region between peaks 1 and 2 consist of conjugates made up of three or more protein molecules. Fractions corresponding to peak 2 were collected and pooled. The elution pattern of these pooled fractions is shown in Fig. 2D. The major species is PE-avidin. Some unreacted phycoerythrin and a small amount of unreacted avidin (the shoulder labeled 4) remained after this fractionation step. The capacity of this PE-avidin preparation for biotin was tested by adding it to biotin-Sepharose beads. These stained beads displayed intense orange-red fluorescence characteristic of phycoerythrin. The prior addition of avidin to biotin-Sepharose beads, or of biotin to the PE-avidin conjugate, blocked the binding of PE-avidin to these beads, as evidenced by the fact that they appeared dark under the fluorescence microscope. Likewise, biotin-Sepharose beads stained with either phycoerythrin or biotinylated phycoerythrin did not fluoresce in the orange-red spectral region. These experiments, as well as the fluorescence-activated cell analyses to be discussed shortly, demonstrate that the PE-avidin conjugate binds specifically to biotin and biotinylated molecules. Furthermore, the quantum yield and emission spectrum of the PE-avidin conjugates are virtually the same as those of native phycoerythrin. A 1 ml sample of 10^{-12} M phycoerythrin (or phycoerythrin conjugate) excited at 520 nm gives a fluorescence signal at 576 nm that is twice as large as the Raman scattering at 631 nm from water. Thus, 10^{-15} mole of phycoerythrin in a standard fluorescence cuvet can readily be detected.

It is of interest to compare the fluorescence intensity of phycoerythrin with that of fluorescein. The fluorescence intensity of a dilute solution of a chromophore is proportional to $\epsilon c Q$, where c is the molar absorbance coefficient at the excitation wavelength, and Q is the fluorescence quantum yield. For excitation at 488 nm (an argon-ion laser line): $\epsilon = 1.28 \times 10^6 \text{ cm}^{-1} \text{ M}^{-1}$ and $Q = 0.82$ for phycoerythrin; and $\epsilon = 8 \times 10^4 \text{ cm}^{-1} \text{ M}^{-1}$ and $Q = 0.9$ for fluorescein. Hence, a solution of phycoerythrin excited at 488 nm has a fluorescence intensity

14.5 as high as that of an equimolar solution of fluorescein. The observed intensity ratio depends also on the efficiency of the detection system with respect to emission wavelength. A phycoerythrin/fluorescein intensity ratio of 10 was measured when equimolar solutions of these substances were flowed through the cell sorter. We estimate that 10^3 molecules of phycoerythrin bound to a cell could be detected in flow cytometry experiments.

IgG-Sepharose beads displayed bright orange-red fluorescence after staining with the phycoerythrin-protein A conjugate, as anticipated, since protein A is known to bind to the F_c portion of mouse $\gamma 2a$ immunoglobulins (27, 28). This staining of the beads was inhibited by the addition of soluble IgG2a. Likewise, the conjugate of phycoerythrin with a monoclonal anti-allotype antibody stained Sepharose beads containing covalently attached target immunoglobulin. These beads did not become fluorescent if soluble target immunoglobulin was added first to the phycoerythrin-antibody conjugate, or if soluble anti-allotype antibody was added first to the beads. Thus, the specific binding characteristics of protein A and of the anti-allotype antibody were retained in their conjugates with phycoerythrin.

The PE-avidin conjugate was used as the red-fluorescent stain in two-color fluorescence-activated cell analyses. We investigated the distribution of Leu antigens (OKT antigens) on the surface of human T-lymphocytes.¹ Leu-1 and Leu-4 are known to be present on all T-cells, whereas Leu-2a and Leu-2b are associated with suppressor and cytotoxic T cells, and Leu-3a and Leu-3b with helper and inducer T cells. The densities of Leu-1, Leu-2a, Leu-3b, and Leu-4 were determined by staining cells with a fluoresceinated antibody specific for one of these antigens and measuring the green fluorescence of these cells. The density of Leu-3a was ascertained by staining T-lymphocytes with biotinylated antibody specific for Leu-3a, followed by PE-avidin, and measuring the red fluorescence. The two-color analysis of T-lymphocytes for Leu-3a and Leu-3b is shown in Fig. 3. The control experiment, in which cells were stained with PE-avidin but not with an antibody, is shown in Fig. 3A. The peak near the origin of this plot arises from the autofluorescence of these cells, inasmuch as unstained cells give the same pattern. Thus, PE-avidin has virtually no affinity for these cells. The result of staining these cells with fluoresceinated anti-Leu-3b is shown in Fig. 3B. Two peaks are evident: the one near the origin arises from cells (~50%) devoid of Leu-3b on their surface, whereas the other comes from cells (~50%) expressing Leu-3b. The green signal is 50 times as bright as the autofluorescence of the Leu-3b negative cells, whereas their red signals are the same. The converse result, shown in Fig. 3C, was obtained when T-lymphocytes were stained with biotinylated anti-Leu-3a, followed by PE-avidin. The red fluorescence of cells containing Leu-3a on their surface is 30 times as high as that of negative cells, whereas the green signals of these two populations are the same. The result of a simultaneous analysis for Leu-3a and Leu-3b is shown in Fig. 3D. The peak near the origin arises from cells that express neither antigen. The other peak comes from cells that contain both Leu-3a and Leu-3b. In other words, every cell that expresses Leu-3a also expressed Leu-3b, and vice versa. This binding is expected, because 3a and 3b are known to be nonoverlapping determinants on the same protein molecule.

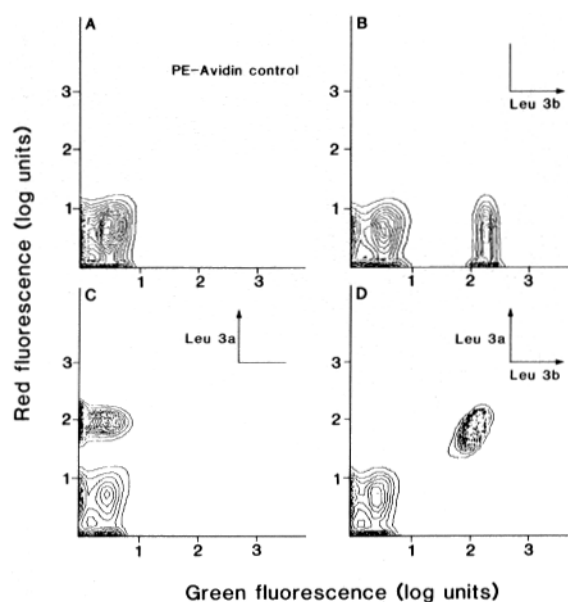


FIGURE 3 Two-color fluorescence-activated cell sorting analysis of human peripheral blood lymphocytes. (A) PE-avidin control; (B) fluorescein-stained for Leu-3b; (C) phycoerythrin-stained for Leu-3a; (D) stained for both Leu-3a and Leu-3b. The stain for Leu-3b was a fluorescein-labeled anti-Leu-3b antibody. The stain for Leu-3a was a biotinylated anti-Leu-3a antibody, followed by PE-avidin.

¹ The identity of Leu and OKT antigens has recently been determined (17): Leu-1 = OKT1; Leu-2 = OKT8; Leu-3 = OKT4; and Leu-4 = OKT 3.

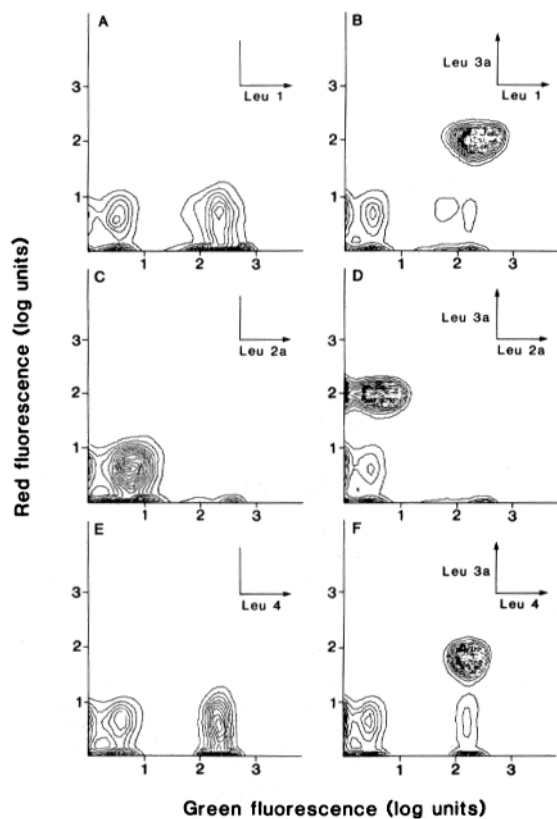


FIGURE 4 Two-color fluorescence-activated cell sorting analyses of human peripheral blood lymphocytes. The patterns on the left side of the figure are for cells stained with fluoresceinated antibody specific for (A) Leu-1, (C) Leu-2a, and (E) Leu-4. The patterns on the right side of the figure are for cells additionally stained with a biotinylated anti-Leu-3a antibody, followed by PE-avidin. Thus, B is stained for Leu-1 and Leu-3a, D for Leu-2a and Leu-3a, and F for Leu-4 and Leu-3a. The pattern for cells stained only for Leu-3a is given in Fig. 3 C.

Two-color fluorescence-activated cell analyses of the distribution of Leu-3a relative to Leu-1, Leu-2a, and Leu-4 on human peripheral blood lymphocytes were also carried out. The profiles of cells stained only with fluoresceinated antibody specific for one of these antigens (green fluorescence) are shown on the left side of Fig. 4, whereas the right side of that figure shows the profiles for cells additionally stained with biotinylated anti-Leu-3a followed by PE-avidin (red fluorescence). The Leu 1 staining pattern (Fig. 4 A) indicates that ~60% of peripheral blood lymphocytes from this donor are T-cells because the Leu-1 antigen is known to be present on all human peripheral T-cells. The profile obtained by also staining for Leu-3a (Fig. 4 B) shows that not all T-cells bear the Leu-3a antigen. About 80% of the T-cells carry Leu-3a and Leu-1. These doubly-positive cells include the helper and inducer T-cell subpopulations. A different result was obtained for the distribution of Leu-2a and Leu-3a. As shown in Fig. 4 C, only 10% of all lymphocytes analyzed express Leu-2a, an antigen known to be associated with the suppressor and cytotoxic T-cell subpopulations. The profile shown in Fig. 4 D demonstrates that a T-cell expresses Leu-2a or Leu-3a but not both antigens. This mutual exclusion is in harmony with the fact that a particular T-cell may be either a suppressor-cytotoxic or a helper-inducer cell, but not both (17, 18). The distribution of Leu-4 (Fig. 4 E) is similar to that of Leu-1, as expected because Leu-4 is known to be present on all human peripheral T-cells

(16, 17). As shown in Fig. 4 F, all cells positive for Leu-3a are also positive for Leu-4.

DISCUSSION

These experiments show that phycobiliprotein conjugates are a valuable class of fluorescent reagents for analyses of molecules and cells. The fluorescence-activated cell analyses depicted in Figs. 3 and 4 demonstrate that phycoerythrin conjugates can be used together with fluorescein-labeled reagents for two-color analyses. The high extinction coefficient of phycoerythrin at 488 nm, the position of an argon-ion laser line, makes it possible to simultaneously excite fluorescein and phycoerythrin with high efficiency. Their fluorescence emission maxima lie at 515 and 576 nm, respectively, and so their emission contributions can readily be separated by appropriate filters. An argon-ion laser and a rhodamine 6G dye laser have recently been used to carry out two-color analyses with fluorescein and Texas Red as labels (R. Hardy, K. Hayaleawa, J. Haaijman, and L. A. Herzenberg, manuscript submitted for publication). An advantage of using phycoerythrin is that a single laser line suffices for two-color analyses. Phycobiliprotein conjugates open up the possibility of carrying out three-parameter analyses with two laser sources. For example, allophycocyanin could serve as the third fluorescent chromophore. In such a three-color experiment, fluorescein and phycoerythrin could be excited by the 488 nm argon-ion line, and allophycocyanin by the 625 nm output of a dye laser (or by a krypton or helium-neon laser). The absorption and emission spectra of phycobiliproteins (Fig. 1) point to the possibility of four-color analyses if C-phycoerythrin conjugates were also employed. The scope and power of fluorescence-activated cell analyses and sorting will undoubtedly be enhanced by the feasibility of detecting multiple distinctive fluorescence signals. For example, changing patterns of cell-surface antigens in differentiation and in disease states could be monitored by multi-colored fluorescence analyses. A case in point is that some malignant T-cell lines express both the Leu-2a and Leu-3a antigens (17). A fluorescein-phycoerythrin two-color analysis of the kind shown in Fig. 4 D would readily detect such aberrant cells. Three and four-parameter cell analyses might be even more revealing.

The bead-staining experiments reported here suggest that phycobiliprotein conjugates are well-suited for fluorescence immunoassays. The fluorescence of femtomole quantities of phycobiliproteins such as phycoerythrin can readily be detected. Furthermore, background fluorescence from body fluids and supporting media, diminishes markedly in going to the red end of the spectrum. The orange-red emission of phycobiliproteins is particularly advantageous in this regard. Our conjugation experiments show that phycobiliproteins can readily be linked by standard coupling reactions to immunoglobulins, protein A, biotin, and avidin. We think it likely that phycobiliproteins can be conjugated with equal facility to lectins, toxins, hormones, growth factors and other biologically interesting molecules for application to a wide range of highly sensitive fluorescence analyses.

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REFERENCES

1. Herzenberg, L. A., and L. A. Herzenberg. 1978. Analysis and separation using the fluorescence-activated cell sorter (FACS). In *Handbook of Experimental Immunology*. 3rd ed. D. M. Weir, editor. Blackwell Scientific Publication, Oxford. Chap. 22. 22.1-22.21.
2. Taylor, D. L., and Y.-L. Wang. 1980. Fluorescently labeled molecules as probes of the structure and function of living cells. *Nature (Lond.)* 284:405-410.
3. Schlessinger, J., Y. Shechter, M. C. Willingham, and I. Pastan. 1978. Direct visualization of binding, aggregation, and internalization of insulin and epidermal growth factor on living fibroblast cells. *Proc. Natl. Acad. Sci. U. S. A.* 75:2659-2663.
4. Koppel, D. E., D. Axelrod, J. Schlessinger, E. L. Elson, and W. W. Webb. 1976. Dynamics of fluorescence marker concentration as a probe of mobility. *Biophys. J.* 16:1315-1329.
5. Glazer, A. N. 1977. Structure and molecular organization of the photosynthetic accessory pigments of cyanobacteria and red algae. *Mol. Cell. Biochem.* 18:125-140.
6. Glazer, A. N. 1981. Photosynthetic accessory proteins with bilin prosthetic groups. In *The Biochemistry of Plants*. Academic Press, Inc., New York. 8:51-96.
7. Yu, M.-H., A. N. Glazer, K. G. Spencer, and J. A. West. 1981. Phycoerythrins of the red alga *Callithamnion*. Variation in phycoerythrobilin and phycourobilin content. *Plant Physiol.* 68:482-488.
8. Barber, D. J. W., and J. T. Richards. 1977. Energy transfer in the accessory pigments R-phycoerythrin and C-phycoerythrin. *Photochem. Photobiol.* 25:565-569.
9. Glazer, A. N., and C. S. Hixson. 1977. Subunit structure and chromophore composition of rhodophytan phycoerythrins. *Porphyridium cruentum* B-phycoerythrin and b-phycoerythrin. *J. Biol. Chem.* 252:32-42.
10. Grabowski, J., and E. Gantt. 1978. Photophysical properties of phycobiliproteins from phycobilisomes: fluorescence lifetimes, quantum yields, and polarization spectra. *Photochem. Photobiol.* 28:39-45.
11. Glazer, A. N., S. Fang, and D. M. Brown. 1973. Spectroscopic properties of C-phycoerythrin and of its α and β subunits. *J. Biol. Chem.* 248:5679-5685.
12. Dale, R. E., and F. W. J. Teale. 1970. Number and distribution of chromophore types in native phycobiliproteins. *Photochem. Photobiol.* 12:99-117.
13. Cohen-Bazire, G., S. Beguin, S. Rimon, A. N. Glazer, and D. M. Brown. 1977. Physicochemical and immunological properties of allophycocyanins. *Arch. Microbiol.* 111:225-238.
14. Ledbetter, J. A., R. V. Rouse, H. S. Micklem, and L. A. Herzenberg. 1980. T cell subsets defined by expression of Lyt-1,2,3 and Thy-1 antigens. *J. Exp. Med.* 152:280-295.
15. Ledbetter, J. A., R. L. Evans, M. Lipinski, C. Cunningham-Rundles, R. A. Good, and L. A. Herzenberg. 1981. Evolutionary conservation of surface molecules that distinguish T lymphocyte helper/inducer and cytotoxic/suppressor subpopulations in mouse and men. *J. Exp. Med.* 153:310-323.
16. Evans, R. L., D. W. Wall, C. P. Patsoucas, F. P. Siegal, S. M. Fikris, C. M. Testa, and R. A. Good. 1980. Thymus-dependent membrane antigens in man: inhibition of cell-mediated lympholysis by monoclonal antibodies to T_{H2} antigen. *Proc. Natl. Acad. Sci. U. S. A.* 78:544-548.
17. Ledbetter, J. A., A. E. Frankel, L. A. Herzenberg, and L. A. Herzenberg. 1981. Human Leu T cell differentiation antigens: quantitative expression on normal lymphoid cells and cell lines. In *Monoclonal Antibodies and T Cell Hybridomas*. G. J. Hammerling, U. Hammerling, and J. F. Kearney, editors. Elsevier/North Holland, New York. In press.
18. Reinherz, E. L., P. C. Kung, G. Goldstein, and S. F. Schlossman. 1979. Separation of functional subsets of human T cells by a monoclonal antibody. *Proc. Natl. Acad. Sci. U. S. A.* 76:4061-4065.
19. Terhorst, C., A. Van Asthoven, E. Teinherz, and S. Schlossman. 1980. Biochemical analysis of human T lymphocyte differentiation antigens T4 and T5. *Science (Wash. D. C.)* 209:520-521.
20. Glazer, A. N., and S. Fang. 1973. Chromophore content of blue-green algal phycobiliproteins. *J. Biol. Chem.* 248:65-662.
21. Bryant, D., A. N. Glazer, and F. A. Eiserling. 1976. Characterization and structural properties of the major biliproteins of *Anabaena* sp. *Arch. Microbiol.* 110:61-75.
22. Green, N. M. 1975. Avidin. *Adv. Protein Chem.* 29:85-133.
23. Heitzmann, H., and F. M. Richards. 1974. Use of the avidin-biotin complex for specific staining of biological membranes in electron microscopy. *Proc. Natl. Acad. Sci. U. S. A.* 71:3537-3541.
24. Jue, R., J. M. Lambert, L. R. Pierce, and R. R. Traut. 1978. Addition of sulfhydryl groups to *Escherichia coli* ribosomes by protein modification with 2-iminothiolane (methyl 4-mercaptobutyrimidate). *Biochemistry*. 17:5399-5406.
25. Carlsson, J., H. Drevin, and R. Axen. 1978. Protein thiolation and reversible protein-protein conjugation. N-Succinimidyl 3-(2-pyridyldithio) propionate, a new heterobifunctional reagent. *Biochem. J. (Tokyo)*. 173:723-737.
26. Oi, V. T., V. M. Bryan, L. A. Herzenberg, and L. A. Herzenberg. 1980. Lymphocyte membrane IgG and secreted membrane IgG are structurally and allotypically distinct. *J. Exp. Med.* 151:1260-1274.
27. Mota, G., V. Ghetie, and J. Sjoquist. 1978. Characterization of the soluble complex formed by reacting rabbit IgG with protein A of *S. aureus*. *Immunochemistry*. 15:639-642.
28. Deisenhofer, J. 1981. Crystallographic refinement and atomic models of a human F₁ fragment and its complex with fragment B of protein A from *Staphylococcus aureus* at 2.9- and 2.8-Å resolution. *Biochemistry*. 20:2361-2370.
29. Oi, V. T., and L. A. Herzenberg. 1979. Localization of murine Igh-1b and Igh-1a (IgG2a): allotypic determinants detected with monoclonal antibodies. *Mol. Immunol.* 16:1005-1017.
30. Mishell, B. B., and S. M. Shiiigi. 1980. Selected Methods in Cellular Immunology. W. H. Freeman and Company, San Francisco.
31. Loken, M. R., D. R. Parks, and L. A. Herzenberg. 1977. Two-color immunofluorescence using a fluorescence-activated cell sorter. *J. Histochem. Cytochem.* 25:899-907.