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Detection of low-density cell-surface molecules using biotinylated fluorescent microspheres

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Biotinylated fluorescent microspheres have been developed as a reagent for studying antigens and receptors expressed at the cell surface. Labeling of antigen or receptor was accomplished by crosslinking biotinylated microspheres through streptavidin to corresponding biotinylated antibodies or ligands. Detection of labeled cells by flow microfluorimetry provided an extremely sensitive means for the analysis and potential manipulation of heterogeneous cell populations. The data indicate that cells bearing fewer than 200 surface antigen-antibody complexes per cell are readily detectable by this approach. Crosslinked to a selected biotinylated peptide immunogen, biotinylated fluorescent microspheres also allowed the labeling and detection of hybridoma cells bearing antigen-specific surface immunoglobulin.

Introduction

The use of fluorescent immunoreagents in combination with flow microfluorimetry to identify, quantify and isolate cells according to their surface antigens and receptors permits the monitoring and manipulation of heterogeneous cell populations at the single-cell level. Hence, flow microfluorimetry [1,2] has been extremely useful in studies of cell differentiation [3,4], plasma membrane receptors [5–7], and genes coding for antigens expressed at the cell surface, including their chromosomal mapping and cloning by expression [8–10]. Due to the limited fluorescence of conventional soluble immunoreagents, however, cells bearing very low densities of antigen or receptor of interest typically escape detection. While it has been estimated by extrapolation that flow microfluorimeters possessing newly developed optics and lasers of high power are capable of detecting cells bearing as few as 1500 fluorescein molecules [11], significantly greater intensities of cell-associated fluorescence are required for conventional microfluorimetry. Previous studies have demonstrated that the detection of rare cells within heterogeneous populations may be facilitated by attaching appropriate immunoreagents to highly fluorescent latex microspheres [12,13]. In order to detect rare cells which, in addition, bear antigens or receptors at exceedingly low densities we now have developed biotinylated fluorescent microspheres as a means for selectively labeling cell surfaces. We have found that biotinylated microspheres used in combination with streptavidin and biotinylated antibodies permit the labeling of cells bearing fewer than 200 antigen-bound IgG molecules, thus extending the sensitivity of flow microfluorimetric detection of

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Abbreviations: SPDP, *N*-succinimidyl-3-(pyridyldithio)-propionate; NHS-biotin, *N*-hydroxysuccinimidobiotin.

low-density cell-surface antigens by one to two orders of magnitude.

Materials and Methods

Albumin-conjugated erythrocytes. Bovine serum albumin was attached to surface sulfhydryl groups of sheep erythrocytes using *N*-succinimidyl-3-(pyridyldithio)propionate (SPDP) [14]. Albumin (10 mg/ml in 0.1 M NaCl/0.07 M $Na_2HPO_4 \cdot 7H_2O$/0.03 M $NaH_2PO_4 \cdot H_2O$ (pH 7.5)) was incubated with SPDP (6.5 mg/ml) for 30 min at 23°C and the thiolated product was isolated by dialysis at 4°C against phosphate-buffered saline (2.7 mM KCl/0.9 mM $CaCl_2$/0.5 mM $MgCl_2 \cdot 6H_2O$/137 mM NaCl/1.4 mM KH_2PO_4/8.1 mM Na_2HPO_4 (pH 7.4)). Erythrocytes (1.25 ml of a 2% suspension) were incubated with dithiothreitol at 35 mM in phosphate-buffered saline for 1 h at 23°C. 200 µl washed, packed cells were then suspended in 0.2 ml phosphate-buffered saline and incubated overnight with 0.2 ml thiolated albumin. The resulting albumin-conjugated erythrocytes were stored for no longer than 1 week in phosphate-buffered saline at 4°C, and were washed prior to use.

Biotinylated anti-albumin IgG. Antibodies to bovine serum albumin were purified from sera of rabbits previously immunized with albumin in the form of haptenic carrier [15]. Purification of antibodies was accomplished by $(NH_4)_2SO_4$ precipitation (50% saturated solution), DEAE-cellulose chromatography [16], and affinity chromatography on immobilized bovine serum albumin (Affi-Gel-10, Bio-Rad) using 4 M guanidine-HCl/50 mM Tris-HCl (pH 7.0) as elution buffer. Biotin was covalently attached to anti-albumin IgG using *N*-hydroxysuccinimidobiotin (NHS-biotin) [17] with IgG and NHS-biotin at final concentrations of $1.6 \cdot 10^{-6}$ M and $8.3 \cdot 10^{-5}$ M, respectively. As determined using an enzyme-linked assay [18] biotinylated anti-albumin IgG retained essentially all of its antigen binding activity, and was precipitated specifically (82%) by avidin [19].

Measurements of biotinylated anti-albumin IgG-albumin complexes or erythrocytes using ^{125}I-labeled protein A. Albumin-conjugated erythrocytes were incubated at $5 \cdot 10^7$ cells/ml with specified dilutions of biotinylated anti-albumin IgG in phosphate-buffered saline/1% normal rabbit serum, for 12 h at 4°C. Following three washes in 10 ml phosphate-buffered saline/0.5% gelatin, $5 \cdot 10^7$ erythrocytes were incubated for 1 h at 23°C in a total volume of 0.5 ml phosphate-buffered saline with ^{125}I-labeled protein A (1.19 µCi/µg) (New England Nuclear) at increasing concentrations. The number of molecules of ^{125}I-labeled protein A specifically bound per cell was determined and served as an estimate of the cell-surface density of anti-albumin IgG-albumin complexes.

Synthesis of biotinylated fluorescent microspheres. Biotinylated fluorescent microspheres were prepared by reacting 0.15 ml of washed, sonicated amino-microspheres (Covalent Technologies, green FX spheres, 1 µm diameter) in 0.75 ml of 0.1 M NaCl/0.07 M $Na_2HPO_4 \cdot 7H_2O$/0.03 M $NaH_2PO_4 \cdot H_2O$ (pH 7.5), with NHS-biotin (0.3 mg in 0.75 ml 25% dimethylformamide in water) for 1 h at 23°C. Washed biotinylated microspheres were stored at 4°C in 1 ml of phosphate-buffered saline/10 mM NaN_3, and were sonicated prior to use.

Biotinylated fluorescent microsphere labeling of albumin-conjugated erythrocytes. Biotinylated fluorescent microsphere labeling of albumin-conjugated erythrocytes was accomplished by first incubating cells ($5 \cdot 10^7$ cells/ml) with specified dilutions of biotinylated anti-albumin IgG for 12 h at 4°C. Cells then were washed three times in 10 ml phosphate-buffered saline/1% normal rabbit serum, incubated at $5 \cdot 10^7$ cells/ml with streptavidin (5 µg/ml) for 2 h at 23°C, and washed again. Approx. $5 \cdot 10^6$ of these cells were admixed with $1 \cdot 10^8$ biotinylated fluorescent microspheres in 96-well tissue culture plates (Costar No. 3596) (total volume, 0.3 ml phosphate-buffered saline/1% normal rabbit serum), sedimented at $250 \times g$ for 10 min at 4°C, and incubated at 4°C for an additional 30 min. Microspheres and cells were transferred with 0.3 ml phosphate-buffered saline/1% normal rabbit serum to 15 ml conical tubes, underlayed with 1.5 ml of Percoll at 41% in phosphate-buffered saline, and centrifuged at $350 \times g$ for 10 min at 4°C. Microspheres at the Percoll interface were removed. Pelleted cells were resuspended in 0.5 ml phosphate buffered saline/1% normal rabbit serum, and were analyzed by flow microfluorimetry (Ortho Cytofluorograph 50 H,

150 comptuer; 5.13 fluorescence gain, linear scale; 20000 cells per analysis). Forward-angle light-scatter was adjusted to allow for discrimination between labeled erythrocytes and unbound microspheres on the basis of size (2.0 gain, linear scale).

Biotinylated fluorescent microsphere labeling of Y5B12 hybridoma cells. Labeling of Y5B12 cells at surface immunoglobulin was accomplished using preassembled biotinylated microsphere-streptavidin-biotinylated peptide immunogen complexes. Biotinylated fluorescent microspheres (0.25 ml) were incubated for 1 h at 37°C with streptavidin (0.01 ml, 1 mg/ml in phosphate-buffered saline/1% bovine serum albumin), washed, resuspended, and sonicated in phosphate-buffered saline/1% bovine serum albumin. Biotinylated peptide then was added (0.05 ml, 0.5 mg/ml) and was incubated with microspheres for 1 h at 37°C. The resulting peptide-coated microspheres were washed and sonicated prior to use. Biotinylated peptide was prepared by incubating peptide (0.25 ml, 1 mg/ml) with NHS-biotin (0.25 ml, 1 mg/ml) for 2 h at 30°C in 0.1 M sodium carbonate–bicarbonate/12.5% dimethylformamide (pH 10.0) and isolating the biotinylated product by chromatography on Sephadex G-15. Flow microfluorimetry and the incubation of cells with microspheres were performed as described above.

Results and Discussion

In testing biotinylated fluorescent microspheres and in assessing their sensitivity, we selected a model system in which bovine serum albumin covalently bound to sheep erythrocytes using SPDP [14] served as a targeted surface antigen. Labeling of surface antigen with microspheres was accomplished in two steps. First, albumin-con-

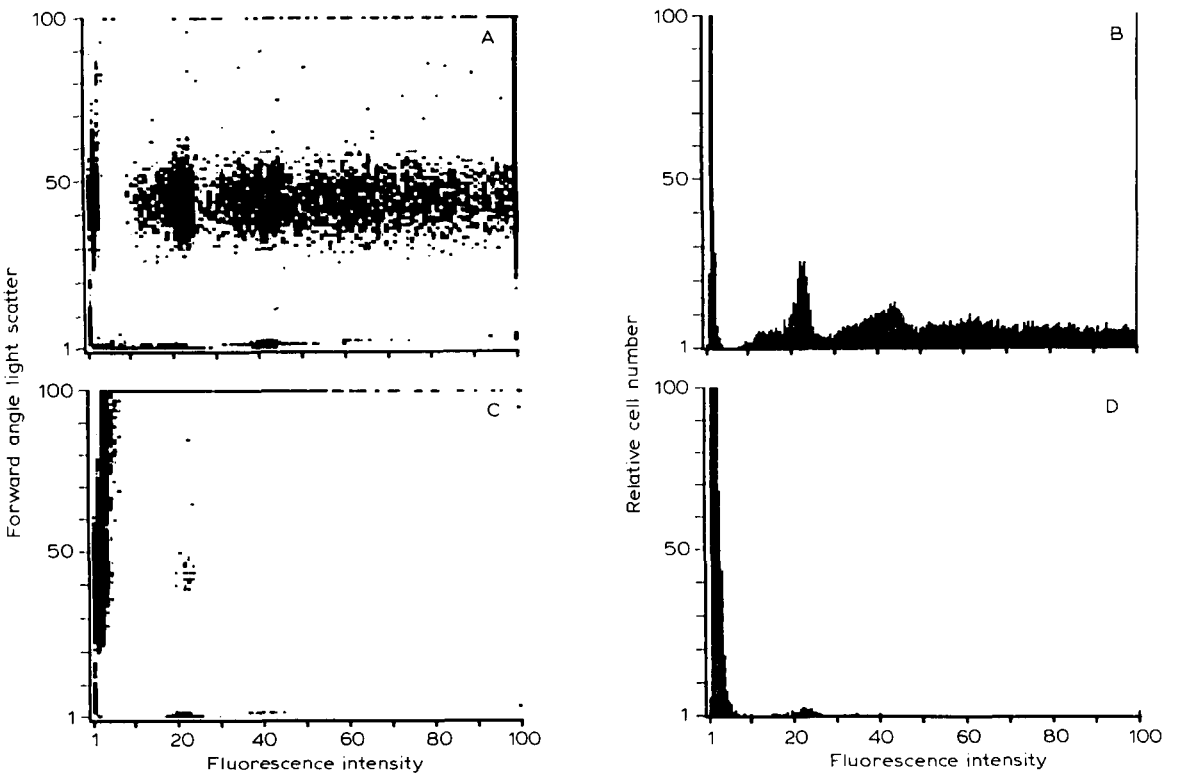

Fig. 1. Labeling of albumin-conjugated erythrocytes with anti-albumin IgG and biotinylated fluorescent microspheres. Following sequential incubations with biotinylated anti-BSA IgG (1 : 50 dilution), streptavidin and biotinylated microspheres, greater than 90% of albumin-conjugated erythrocytes were fluorescently labeled as measured by flow microfluorimetry (panels A and B). As assessed by the co-incubation of cells with biotinylated fluorescent microspheres and excess (+)-biotin (0.05 mg/ml) (panels C and D), nonspecific binding of microspheres to cells was less than 1% of total.

jugated erythrocytes were incubated with affinity-purified biotinylated antibodies to albumin. Biotinylated fluorescent microspheres then were crosslinked through streptavidin to biotinylated antibody-albumin complexes at the cell surface. In initial experiments using antibody at a dilution of 1 : 50 ($2.5 \cdot 10^{-8}$ M IgG), greater than 90% of these cells were labeled, with 10% bearing greater than five microspheres per cell (Fig. 1, panels A and B). As assessed by the co-incubation of cells either with biotinylated microspheres and (+)-biotin (0.1 mg/ml) (Fig. 1, panels C and D) or with biotinylated anti-albumin IgG and bovine serum albumin (0.1 mg/ml) (not shown), nonspecific binding of biotinylated fluorescent microspheres to cells was less than 1% of total.

By the following means, use of the albumin-conjugated erythrocyte model antigen system provided an estimate of the lower limit of the density of cell-surface antigen detectable by biotinylated fluorescent microsphere labeling. Erythrocytes bearing albumin were first incubated with specified decreasing concentrations of biotinylated anti-albumin IgG. The average number of anti-

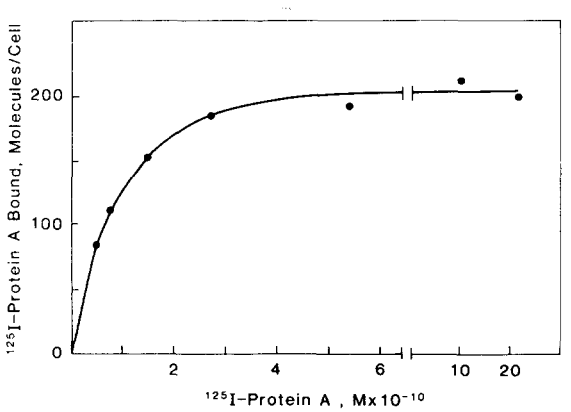

Fig. 2. Binding of ^{125}I-labeled protein A to IgG-albumin complexes on erythrocytes. Erythrocytes bearing albumin were incubated with biotinylated anti-albumin IgG at a dilution of 1 : 100, washed, and incubated with increasing concentrations of ^{125}I-labeled protein A. The mean number of molecules of ^{125}I-labeled protein A specifically bound per erythrocyte (approx. 200) served as a direct estimate of the mean density of IgG-albumin complexes formed using this dilution of antibody. At all concentrations, nonspecific binding of ^{125}I-labeled protein A was less than 5% of total as determined following the co-incubation of erythrocytes with antibody and excess bovine serum albumin (0.1 mg/ml).

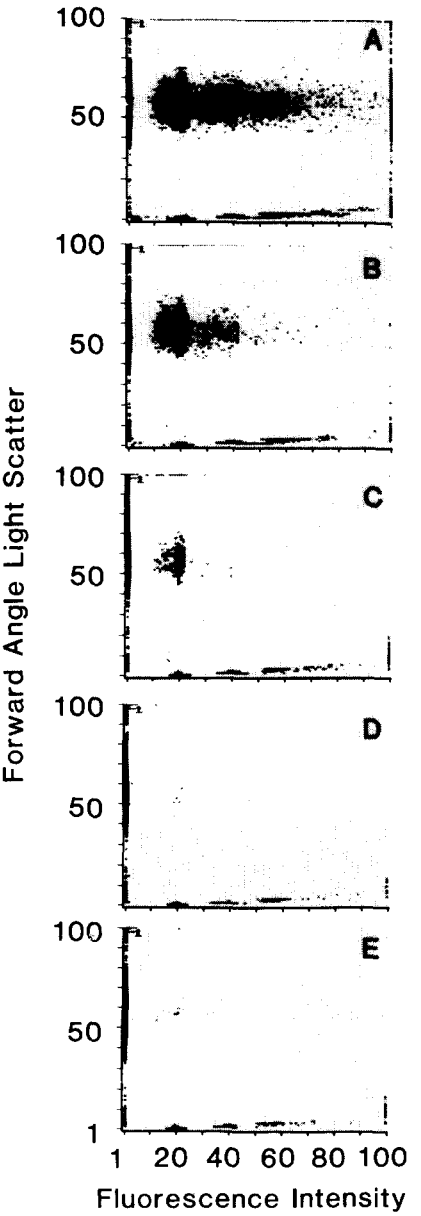

Fig. 3. Biotinylated fluorescent microsphere labeling of erythrocytes bearing low densities of biotinylated anti-albumin IgG-albumin complexes. Dilutions of biotinylated anti-albumin IgG used in albumin-conjugated erythrocyte labeling were panel A, 1:100; B, 1:500; D, 1:100; E, 1:100. The molar concentrations of antibody corresponding to these dilutions are 1:100, $1.25 \cdot 10^{-8}$ M; 1:500, $2.5 \cdot 10^{-9}$ M; 1:1500, $8.3 \cdot 10^{-10}$ M. Controls for nonspecific binding were panel D, cells incubated with biotinylated anti-albumin IgG in the presence of excess albumin (0.1 mg/ml); panel E, cells incubated with biotinylated fluorescent microspheres in the presence of excess (+)-biotin (0.05 mg/ml).

body-albumin complexes formed per cell then was measured directly using ^{125}I-labeled protein A of known specific radioactivity and Fc-region binding activity (Fig. 2). Assuming monovalency of IgG for protein A, incubation with antibody at a dilution of 1 : 100 yielded cells bearing approx. 200 IgG-albumin complexes. Densities of IgG-albumin complexes resulting from the incubation of cells with antibody at dilutions of 1 : 500 or 1 : 1500 were too low for detection by the ^{125}I-labeled protein A binding assay. Therefore, these densities were estimated both by extrapolation, and by a more conservative bimodal model (see below). When labeled using streptavidin and biotinylated fluorescent microspheres, those populations of albumin-conjugated erythrocytes incubated with biotinylated albumin antibodies at dilutions of 1 : 100, 1 : 500, and 1 : 1500 were shown by flow microfluorimetry to contain labeled cells at frequencies of 51.7%, 23.2% and 7%, respectively (Fig. 3, panels A–C). Binding of antibody and of biotinylated microspheres to cells was specific: the co-incubation of excess albumin with antibody and cells (Fig. 3, panel D), or of excess (+)-biotin with microspheres and cells (Fig. 3E), reduced cell-associated fluorescence to background levels.

From these data, two estimates of the density of surface antigens detectable with biotinylated fluorescent microspheres can be made according to two alternative assumptions. These estimates are necessary, since the direct measurement of IgG-albumin complexes using ^{125}I-labeled protein A was not feasible at densities below 200 IgG molecules per cell. In the first instance, it is assumed that the mean density of IgG-albumin complexes occurring on erythrocytes decreases proportionally with the dilution of antibody employed. Incubation of cells with biotinylated anti-albumin IgG at a dilution of 1 : 100 resulted in approximately 200 IgG-albumin complexes per cell (Fig. 2). Accordingly, incubations with antibody at dilutions of 1 : 500 and 1 : 1500 are predicted to yield cells possessing mean densities of 40 and 13 IgG-albumin complexes per cell, respectively. Flow microfluorimetry using biotinylated fluorescent microspheres detected some of the cells incubated with antibody at each of these dilutions (Fig. 3). However, it is possible that in each cell population, those cells labeled with microspheres possessed above-average densities of immune complexes. A second estimate of the sensitivity of biotinylated fluorescent microspheres which accounts for this possibility can be made through an alternative assumption. As stated, the mean density of IgG-albumin complexes on cells incubated with antibody at a dilution of 1 : 100 was measured directly at 200 per cell. Approx. 51% of these cells were labeled by biotinylated microspheres. By the most conservative interpretation in which only fluorescent cells are assumed to bear biotinylated anti-albumin IgG-albumin complexes and nonfluorescent cells are assumed to bear no biotinylated anti-albumin IgG-albumin complexes, the maximal mean density of IgG-albumin complexes for the fluorescent subpopulation is calculated at 200/0.517, i.e., 387 per cell. Therefore it is estimated that 100% of those cells bearing an average of 387 complexes per cell were detected. Lack of additional information concerning the distribution of the density of IgG-albumin complexes among cells precludes the assignment of a more precise limit for the sensitivity of biotinylated fluorescent microspheres in cell surface antigen detection. However, the above analyses of available data indicate that cells bearing approx. 200 antigen molecules, and perhaps less, are detected by this technique.

Several additional features of the biotinylated fluorescent microsphere assay for cell surface molecules merit discussion. The high sensitivity of these microspheres is attributable, in part, to their high capacity for the attachment of biotinylated reagents. Studies using ^{125}I-labeled streptavidin and [^{3}H]biotin (not shown) indicated that approx. 10^4 molecules of a selected biotinylated probe can be specifically crosslinked to each microsphere. This high probe density maximizes the probability of a sphere binding to a low-density cell surface molecule. The biotinylated probe may correspond to any of a variety of biotinylated reagents including primary or secondary antibodies, protein A, or receptor ligands. For example, immunogen covalently coupled to fluorescent microspheres previously has been demonstrated to provide sufficient sensitivity for the detection of hybridoma surface immunoglobulins not detectable using soluble fluorescent immunogen and flow microfluorimetry [13]. Using biotinylated fluorescent microspheres in combination with biotinylated immunogen, we

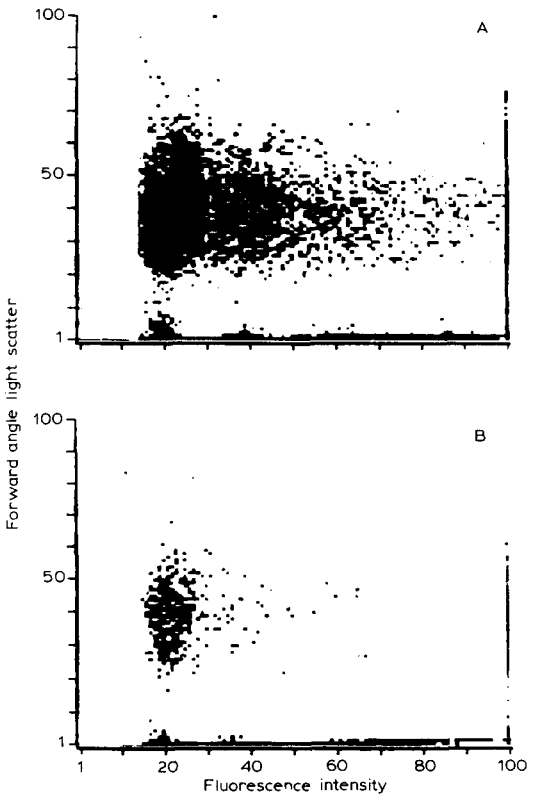

Fig. 4. Labeling of surface immunoglobulin of Y5B12 hybridoma cells using biotinylated peptide immunogen cross-linked to biotinylated fluorescent microspheres. Panel A: flow microfluorimetric analysis of Y5B12 cells labeled with pre-assembled biotinylated peptide immunogen-streptavidin-biotinylated microspheres. Panel B: labeling of Y5B12 cells in the presence of excess underivitized peptide (0.01 mg/ml). Autofluorescence due to unlabeled cells was gated.

have labeled surface immunoglobulin of the cell line Y5B12 (Fig. 4). Y5B12 cells are hybridomas derived from a fusion between P3X-63Ag8653 murine myeloma cells and splenocytes isolated from $CB6F_1$ mouse immunized with a synthetic peptide [15] corresponding to the putative 26 amino acids of the amino-terminus of the mammalian glycoprotein hormone, erythropoietin. These cells have been shown to secrete IgG_1 antibodies to peptide (unpublished data). In the present analysis, fluorescent microspheres were preassembled as biotinylated microsphere-streptavidin-biotinylated peptide complexes prior to incubation with cells (see Materials and Methods). In flow microfluorimetry, 34% of Y5B12 cells bound peptide-conjugated microspheres, with 23% binding two or more microspheres per cell (Fig. 4A). Levels of nonspecific binding were low, with fewer than 5% of either irrelevant cells (e.g., P3X-63Ag8653 cells, not shown) or Y5B12 cells binding single microspheres in the presence of underivitized peptide (Fig. 4B). Y5B12 cells labeled using biotinylated peptide and fluorescein-labeled streptavidin could not be detected by flow microfluorimetry.

In flow microfluorimetric analyses, the binding of each microsphere to cells increased the intensity of cell-associated fluorescence by discrete intervals (Figs. 1, 3 and 4). Consequently, labeling with biotinylated fluorescent microspheres should improve the resolution and isolation of subpopulations of cells possessing relatively small differences in antigen or receptor densities. When labeled with conventional soluble fluorescent reagents, such subpopulations typically display only minor differences within a continuous spectrum of fluorescence intensities [8–10]. While avidin-conjugated fluorescent microspheres have been prepared previously by a more complex method [20], neither the effects of inherent multiple chemical modifications of avidin on its affinity and specificity for biotin binding nor the limits of sensitivity of these acetylated-avidin microspheres in detecting cell surface antigens have been assessed. In contrast, biotinylated fluorescent microspheres are synthesized through a single reaction between fluorescent aminomicrospheres and *N*-hydroxysuccinimidobiotin, and are coupled directly to unmodified streptavidin. Given their flexible design and demonstrated sensitivity in detecting cell-surface molecules, biotinylated fluorescent microspheres and their derivatives should find wide application in research and clinical laboratories. The generalized approach towards attaching active biotinylated reagents to polymeric microspheres also should be of value in the further development of microspheres used in cell separation [21] and in drug delivery systems [22].

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References

1 Shapiro, H.M. (1983) Cytometry 3, 227–243
2 Cambier, J.C. and Monroe, J.G. (1983) Methods Enzymol. 103, 227–245
3 Loken, M.R. and Stall, A.M. (1982) J. Immunol. Methods 50, R85–R112
4 Voet, L., Krefft, M., Mairhofer, H. and Williams, K.L. (1984) Cytometry 5, 26–33
5 Bohn, B. (1980) Mol. Cell. Endocrinol. 20, 1–15
6 Murphy, R.F., Powers, S., Cantor, C.R. and Pollack, R. (1984) Cytometry 5, 275–280
7 Newman, R., Domingo, D., Trotter, J. and Trowbridge, I. (1983) Nature 304, 643–646
8 Kamarck, M.E., Barbosa, J.A., Kuhn, L., Messer-Peters, P.G., Shulman, L. and Ruddle, F.H. (1983) Cytometry 4, 99–108
9 Stanners, C.P., Lam, T., Chamberlain, J.W., Stewart, S.S. and Price, G.B. (1981) Cell 27, 211–221
10 Kavathas, P., Sukhatme, V.P., Herzenberg, L.A. and Parnes, J.R. (1984) Proc. Natl. Acad. Sci. USA 81, 7688–7690
11 Hoffman, R.A., Burger, D.E. and Brown, M.C. (1984) Poster presented at the Tenth Conference of Analytical Cytology, June 3, 1984, Pacific Grove, CA. Publication available through Ortho Diagnostics Systems, Inc., Westwood, MA, U.S.A.
12 Cupp, J.E., Leary, J.F., Cernichiari, E., Wood, J.C.S. and Doherty, R.A. (1984) Cytometry 5, 138–144
13 Parks, D.R., Bryan, V.M., Oi, V.T. and Herzenberg, L.A. (1979) Proc. Natl. Acad. Sci. USA 76, 1962–1966
14 Jou, Y.-H., and Bankert, R.B. (1981) Proc. Natl. Acad. Sci. USA 78, 2493–2496
15 Sue, J.M. and Sytkowski, A.J. (1983) Proc. Natl. Acad. Sci. USA 80, 3651–3655
16 Goding, J.W. (1983) Monoclonal Antibodies: Principles and Practice, pp. 114–115, Academic Press, New York
17 Wagener, C., Clark, B.R., Rickard, K.J. and Shively, J.E. (1983) J. Immunol. 130, 2302–2307
18 Douillard, J.Y. and Hoffman, T. (1983) Methods Enzymol. 92, 169–174
19 Clark, B.R. and Todd, C.W. (1982) Anal. Biochem. 121, 257–262
20 Kaplan, M.R., Calef, E., Bercovici, T. and Gitler, C. (1983) Biochim. Biophys. Acta 728, 112–120
21 Molday, R.S. (1984) in Cell Separation, Methods and Selected Applications (Pretlow, T.G. and Pretlow, T.P., eds.), pp. 237–263, Academic Press, New York
22 Tritton, T.R. and Wingard, L.B. (1984) in Microspheres and Drug Therapy (Illum, D.L., McVie, J.G., Tomlinson, E., eds.), pp. 129–138, Elsevier, Amsterdam