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THE SELECTIVE DETECTION OF CELL SURFACE DETERMINANTS BY MEANS OF ANTIBODIES AND ACETYLATED AVIDIN ATTACHED TO HIGHLY FLUORESCENT POLYMER MICROSPHERES

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Procedures are described for the synthesis of 500 Å-diameter polymer microspheres containing a novel fluorescent cross-linking agent. These microspheres have very high fluorophore concentration without quenching of the fluorescence and show very low nonspecific interaction with cells. When monoclonal anti-Thy-1.2 is attached to the fluorescent microspheres, specific binding results in 10^4 spheres being attached per thymocyte while non-specific binding is less than 1%. Similar values are obtained for an indirect staining procedure. The high non-specific binding of cationic avidin to negative cell surfaces is shown to be decreased to negligible levels by acetylation of the amine groups of the protein without decreasing its high-affinity binding to biotin. The use of acetyl-avidin (p*I* = 6.7) directly, or when attached to fluorescent microspheres, resulted in a highly selective detection of biotinyl groups on the erythrocyte or lymphocyte cell surface. Attachment of biotinyl groups to the hinge carbohydrates of antibodies did not affect their specificity. It allowed their detection by means of microspheres-acetyl-avidin conjugates.

Introduction

The quantitative determination of cell surface receptors is essential for the study of many cellular functions. It would be desirable to have available methods that would allow the quantitative estimate of the average receptor-content per cell and, in addition, the frequency distribution of the receptors within a cell population. Furthermore, it would be desirable to have a method that assays for receptor accessibility at the cell surface. It is likely that different receptors may be present at varying depth within a complex cell surface. Only those most exposed may play a role in cell-cell recognition.

Quantification of receptors has usually been approached by means of radioactive ligands. Such methods estimate only the average number of receptors in a cell population. The development of the fluorescence activated cell sorter opened up possibilities of using fluorescence for quantitative evaluation of surface receptors , yielding, at the same time, information about receptor distribution in a cell population. A critical discussion of flow cytometry as an approach for the quantitative analysis of receptor-ligand interaction has been recently published by Bohn [1]. The sensitivity of this method is limited by the number of fluorescent molecules that can be introduced per ligand without its inactivation and by the extent of non-specific binding, e.g., only three to four molecules of fluorescein can be attached to an antibody molecule without losing activity.

The approach we have used to overcome these

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Abbreviation: Ig, immunoglobulin.

problems has been pioneered by Rembaum and colleagues [2,3]. It involves attachment of the ligand to a polymer that contains a high concentration of fluorophore. These ligand-polymer complexes should allow a significant increase in the fluorescence signal to noise ratio. Such a polymer should have minimal non-specific interaction with cells and a high fluorophore content without leading to quenching or non-specific binding. Also, it should allow for facile coupling of ligands without destruction of their specificity.

In this report we present the synthesis and application of fluorescent polymeric microspheres. We have synthesized a fluorescein-based polymerizable cross-linking agent which allows the incorporation of 700 fluorescein molecules per sphere of 500 Å-diameter. Non-specific binding was less than 1%. We also present our attempts to find a universal ligand by using acetylated avidin and biotinylated antibody. Acetylated avidin, being less basic than avidin, shows low non-specific binding to cells while retaining its capacity to bind biotin. A novel method of attaching biotin to monoclonal antibodies allows the use of microspheres-acetylavidin conjugates for selective surface labeling.

Materials

Diallylamine (Merck) and methylmethacrylate (Fluka) were distilled before use. Paraformaldehyde (BDH), fluorescein sodium salt (BDH), *N,N'*-methylenebisacrylamide (Eastman Kodak), avidin (Sigma) and biotin (Sigma) were used. Sodium dodecyl sulphate and ammonium persulphate (BDH) were of highest purity. All other chemicals were of highest purity. Mouse monoclonal anti-Thy-1.2 and anti-Thy-1.1 were obtained as a gift from Dr. Z. Eschar. C57Bl/6J and AKR/J mice were inbred strains from the Weizmann Institute Bioterium. Freshly withdrawn human erythrocytes and mice thymocytes were used.

Methods

Synthesis of di(methylene diallylamine) fluorescein diacetate. 2 g (4.8 mM) fluorescein diacetate [4], 0.5 g paraformaldehyde (17 mM calculated as formaldehyde), were mixed in 6 ml ethanol with 1.6 ml (12 mM) diallylamine, 40 μl concentrated HCl was added and the reaction mixture refluxed for 2 h. After cooling, the mixture was filtered and the solution precipitated with 30 ml of water. The product was recovered by filtration and recrystallized from water/ethanol (2 : 1, by volume). Yield 60%, m.p. 151°C. The product is non-fluorescent with a maximum absorption peak at 500 nm. It has been characterized by NMR (not shown).

Synthesis of 500 Å-diameter microspheres. 15 g methylmethacrylate, 0.07 g di(methylene diallylamine) fluorescein diacetate, 0.8 g bisacrylamide, 1 ml 10% sodium dodecyl sulfate, 0.4 ml 5% ammonium persulfate and 82 ml double-distilled water were placed in a glass ampule. The biphasic mixture was flushed with nitrogen at 4°C and the ampule was sealed. The ampule was gently rotated in a water bath and heated to 75°C for 1 h, then 98°C for another hour. The resulting milky suspension was filtered through Whatman No. 1 paper. Generally, more than 99% passed through the filter.

Reaction with hydrazine hydrate or ethylenediamine was then carried out, based on the procedure described by Inman and Dintzis [5]. Hydrazide-microspheres were obtained by mixing one volume of 98% hydrazine hydrate with two volumes of microspheres suspension and reacting at 47°C for 24 h. To obtain ethylenediamine-microspheres, 99% ethylenediamine was reacted with microspheres suspension 1 : 1 by volume at 90°C for 24 h. Both derivatives were dialyzed extensively against double-distilled water and stored with 0.02% NaN_3 at 4°C.

Coupling of ligand to microspheres. 20 μl of 2.5% glutaraldehyde solution was added to 0.5 ml microspheres suspension 7 mg/ml. After 30 min reaction at room temperature with stirring, the suspension was passed on a Sepharose G-25 column (1 × 30 cm) equilibrated with 10 mM phosphate buffer, pH 7.4. Phosphate-buffered saline concentrate times 20 was added to the eluted microspheres peak to isotonicity. Protein (0.5–1 mg, unless otherwise stated) was added and the reaction was allowed to proceed for 150 min. Glycine was added to a final concentration of 0.2 M and sodium cyanoborohydride ($NaCNBH_3$) to 1 mM and the reaction continued 16 h at 4°C.

Before use, microspheres were passed on a Sepharose 4B-CL column equilibrated with 0.1% gelatin in phosphate buffered saline.

Staining of cells with microspheres-ligand conjugate. Microspheres eluting in the fluorescent peak of Sepharose 4B-CL were used. 250 μl microspheres-protein conjugate (1.4–3 mg/ml) were added to $2 \cdot 10^7$ cells (200 μl) in phosphate-buffered saline containing 2% bovine serum albumin. After gentle shaking for 1 h at room temperature, cells were washed twice with 0.5% bovine serum albumin in phosphate-buffered saline, once with phosphate-buffered saline. Samples analyzed by the cell sorter were fixed with paraformaldehyde[6].

Iodination of microspheres. 0.3 ml microspheres suspension 35 mg/ml were adjusted to pH 8–8.5 with 0.1 M Na_2HPO_4. The suspension was then transferred to a tube containing 70 μCi of Bolton and Hunter reagent from which solvent was evaporated. The reaction was then carried out exactly as for proteins [8]. To these iodinated microspheres, protein was coupled as described above.

Biotinylation of antibody. Monoclonal anti-Thy-1.2 was precipitated with 40% saturated ammonium sulfate and re-dissolved in phosphate buffered saline. To 0.2 mg (0.2 ml) of the immunoglobulin, $NaIO_4$ was added to a final concentration of 1 mM. After reacting for 30 min at 0°C, the protein was passed on Sepharose G-25. To the pooled protein peak, 1.5 mg biotin hydrazide was added [7] and reacted for 30 min at room temperature. Then, 1 mM $NaCNBH_3$ was added and reduction carried out for 16 h at 4°C. The biotinyl-antibody was passed on a Sephadex G-25 column. Repeated passage on Sephadex G-25 was carried out just before use.

Acetylation of avidin. 67 μg of avidin in 0.15 ml of 0.1 M phosphate buffer, pH 8.0, were added to a 3 mg/ml solution of acetyl-*N*-hydroxysuccinimide ester in ethanol. After 30 min reaction at room temperature, the product was passed on Sephadex G-25.

Analysis of fluorescence in the fluorescence activated cell sorter. A FACS II Becton Dickinson Sorter was used. Excitation by the 488 nm line of an argon ion laser was used. Three 530 nm long-path filters were used at the photomultiplier entrance to remove scattered light.

Results

Synthesis of fluorescent microspheres

We have synthesized a cross-linking agent, di(methylenediallylamine)fluorescein diacetate, which is a non-fluorescent molecule but becomes highly fluorescent during the microspheres synthesis due to its deacylation. The scheme of the synthesis is shown in Fig. 1. The synthesis of the fluorescent microspheres was essentially carried

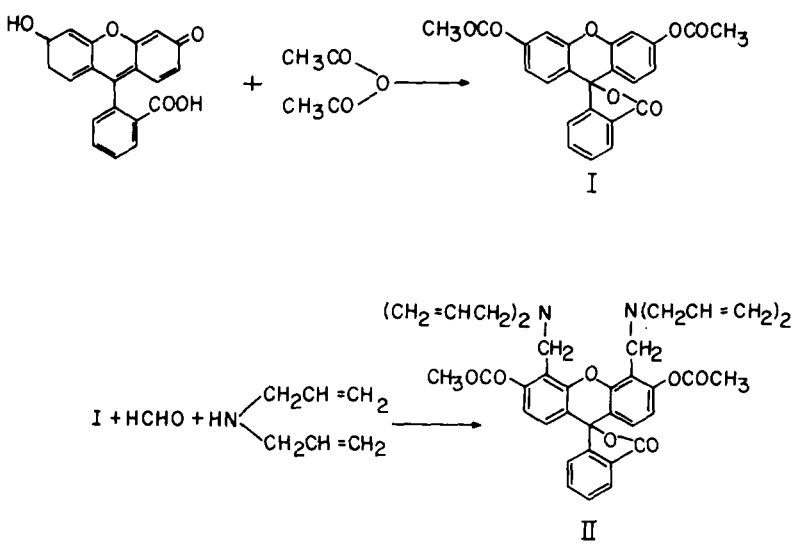

Fig. 1. Synthesis of a fluorescent cross-linking molecule, di(diallylamine) fluorescein.

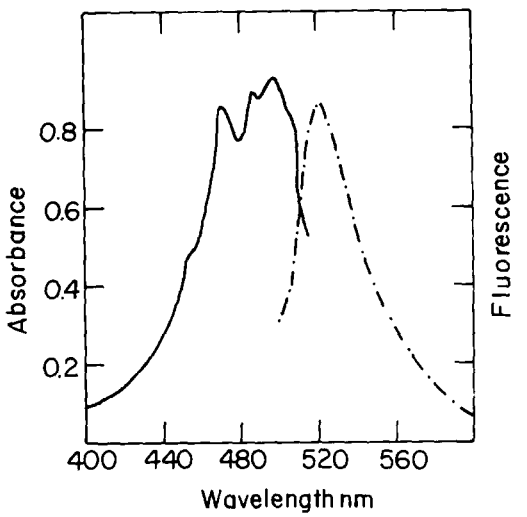

Fig. 2. Absorption and emission spectra of the fluorescent polymeric microspheres.

out in two steps. In the first step, microspheres were synthesized by emulsion polymerization as described in Methods. These were then reacted with either ethylenediamine to yield amine surface groups or with hydrazine hydrate to give hydrazide groups. The use of *N,N'*-bisacrylamide and di(methylenediallylamine) fluorescein, which are non-labile to strong bases, allowed for this second step conversion of surface groups. The hydrazine hydrate treated microspheres were not stable in 0.15 M NaCl and readily aggregated. On the other hand, treatment with ethylenediamine resulted in the conversion of part of the esters into carboxyl groups by base catalyzed hydrolysis. The presence of carboxyls was verified by acid base titration (not shown). The amine-microspheres formed a stable suspension in 0.15 M NaCl and thus were used throughout the experiments described in this work. The determination of the fluorescence yield of the fluorescent microspheres was performed by comparing the fluorescence of the microspheres suspension with that of solutions of fluorescein sodium at 10^{-6}–10^{-8} M and neutral pH, where quenching is negligible. On that basis, a value of $2.5 \cdot 10^{-4}$ M fluorescein equivalents was obtained for a suspension of 35 mg/ml. Since the density of the polymeric microspheres was determined on Percoll gradients to be 1.20, the fluorescein-equivalent concentration in the polymer matrix is 8.6 mM. The absorption and emission spectra of the microspheres are shown in Fig. 2.

Coupling of protein to microspheres

Typically fluorescent microspheres of 500 Å diameter were used. Proteins were coupled by the two-step glutaraldehyde procedure similar to that of Trenynck and Avrameas [9] (Fig. 3 and Methods). The excess of glutaraldehyde added in the first step was removed on a Sephadex G-25 column. The microspheres appeared as a bright fluorescent peak in the void volume. These were then

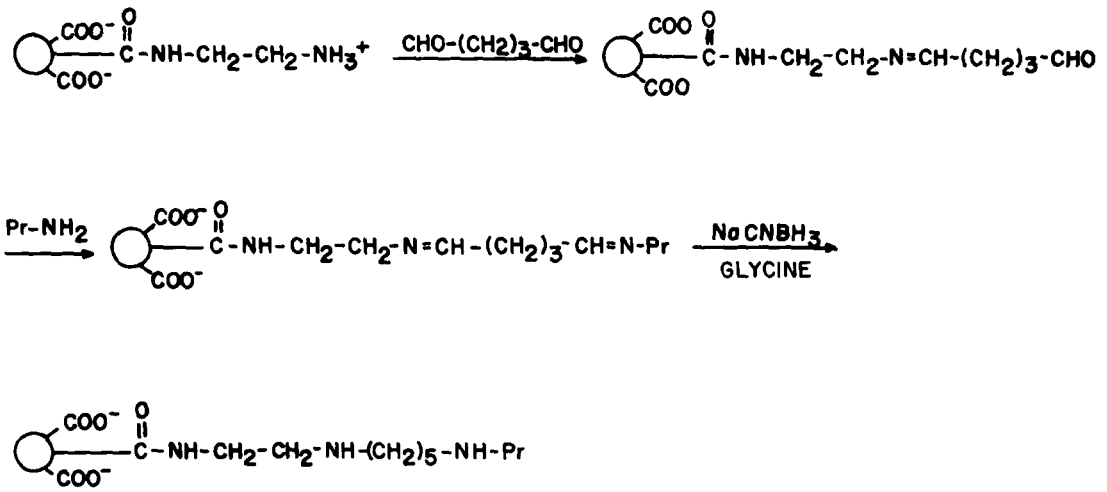

Fig. 3. Derivatization of fluorescent polymeric microspheres.

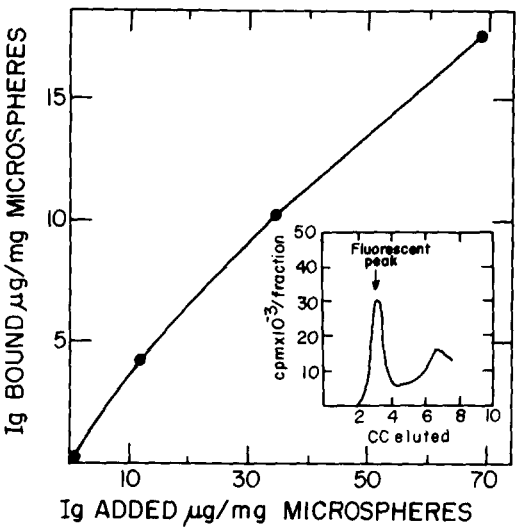

Fig. 4. Binding curve of anti-Thy-1.2 to microspheres. ^{125}I-labeled anti-Thy-1.2 was attached to microspheres, under the conditions described in Methods, at increasing amounts of antibody. Samples were passed on 10 ml Sepharose 4B columns equilibrated with 0.1% gelatin in phosphate-buffered saline. Binding was calculated from the radioactivity eluting with the fluorescent peak. The insert shows the separation on Sepharose 4B of the sample corresponding to 11.4 µg Ig added per mg polymer, resulting in 39% binding.

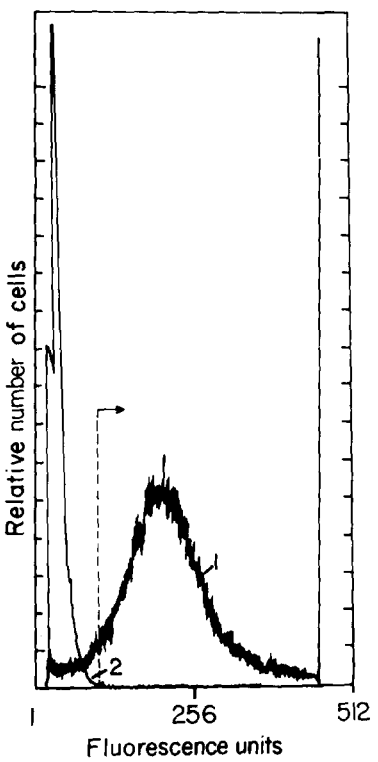

Fig. 5. Indirect staining of thymocytes with microspheres-goat-anti-mouse. C57Bl/6J thymocytes were reacted for 1 h at 4°C with 50 µl at 1:50 dilution of monoclonal anti-Thy-1.2 (1) or anti-Thy-1.1 (2). Cells were washed three times with phosphate-buffered saline and stained with microspheres-goat anti-mouse conjugates as described in Methods.

coupled to protein to form Schiff bases which were reduced by sodium cyanoborohydride. The remaining free aldehyde groups were blocked with glycine to increase the negative charge of the polymer. Excess unbound protein was removed immediately before use by passage through a Sepharose 4B column. The microspheres-protein conjugate appeared in the void volume while the free protein could be recovered in the included volume (Fig. 4, insert).

Fig. 4 shows a typical covalent binding curve of anti-Thy-1.2 to microspheres. The number of protein molecules bound per microsphere varied from one to ten depending on the protein and the concentrations used.

Cells staining with microspheres-antibody conjugates. Direct and indirect procedures

It was found that antibody attached to microspheres could be used for cell staining essentially by the same procedures used for soluble antibodies. Excess of microspheres-antibody could be removed by sedimentation of the cells, while unattached conjugate did not sediment. Two procedures were tested. In the direct method, an antibody recognizing a surface determinant was attached to the microspheres. Alternatively, an indirect procedure was used involving attachment to the microspheres of goat anti-mouse immunoglobulin. These conjugates were then added to cells to which antibody was bound.

As a test system, antibodies against mouse thymocytes Thy-1.2 or Thy-1.1 determinants were used. The distribution of fluorescence was analyzed by means of the fluorescence activated cell sorter. Scatter-gating was used to eliminate dead cells which attach large amounts of polymer non-specifically.

In Figs. 5 and 6 are presented fluorescence

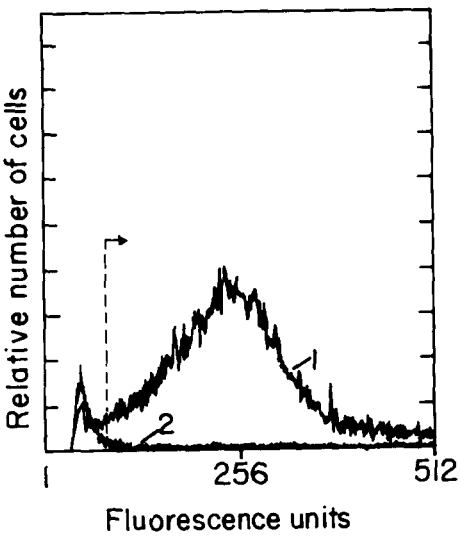

Fig. 6. Direct labeling of thymocytes with microspheres-anti-Thy-1.2 conjugates. C57Bl/6J thymocytes were labeled with microspheres-anti-Thy-1.2 (1) or microspheres-anti-Thy-1.1 conjugates (2) as described in Methods.

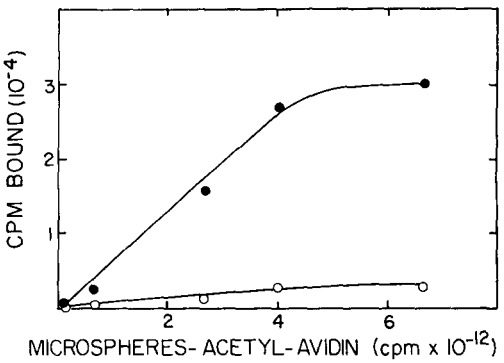

Fig. 7. Binding curve of ^{125}I-labeled microspheres-acetyl-avidin to biotinylated erythrocytes. ^{125}I-labeled microspheres-acetyl-avidin was $6.7 \cdot 10^6$ cpm/mg polymer. The indicated amounts of microspheres-acetyl-avidin were added to $5 \cdot 10^7$ biotinylated erythrocytes in 0.5 ml 2% bovine serum albumin in phosphate-buffered saline. The reaction was allowed to proceed for 1 h at room temperature, with gentle shaking. Cells were washed twice with 0.5% bovine serum albumin in phosphate-buffered saline and counted. Binding was carried out in the absence (●) or presence (○) of 50 μg free biotin, which were added to microspheres-acetyl-avidin 10 min prior to their reaction with cells.

histograms of mouse thymocytes stained by the indirect and direct methods, respectively. In both direct and indirect labeling the ratio of mean channels of sample and control is 30 : 1. In the indirect labeling above the indicated channel (Fig. 5), more than 85% of the cells were stained compared to 1% in the control. In the direct labeling (Fig. 6), more than 73% were labeled with 0.5% stained in the control.

Viability of the cells was not affected by the microspheres-labeling procedure as determined by Trypan blue dye exclusion.

TABLE I

BINDING OF ACETYL-AVIDIN TO BIOTINYLATED ERYTHROCYTES

The acetylation reaction was performed as described in Methods. 15000 cpm of avidin or avidin derivatives ($1.6 \cdot 10^7$ cpm/mg) were added per 10^8 erythrocytes (10% suspension in phosphate-buffered saline containing 0.1% gelatin). Binding was carried out at 20°C for 1 h. When free biotin was present, 50 μg were added to each avidin sample before its addition to cells and pre-incubated for 10 min. Isoelectric points were determined by isolectric focusing. After binding, cells were washed three times with phosphate-buffered saline containing 0.1% gelatin.

No.	μmol of acetyl-*N*-hydroxy succinimide ester added per μmol avidin	Isoelectric point	Avidin binding		Avidin binding in the presence of free biotin		Ratio of binding with and without biotin
			cpm	%	cpm	%	
1	360	4.7	500	3.3	110	0.7	4.5
2	36	n.d. [a]	8000	53.0	110	0.7	73.0
3	18	6.7	11000	73.0	150	1.1	73.0
4	0	> 8.2 [b]	12000	80.0	1300	8.6	90

[a] Not determined.
[b] p*I* = 10.5 [16].

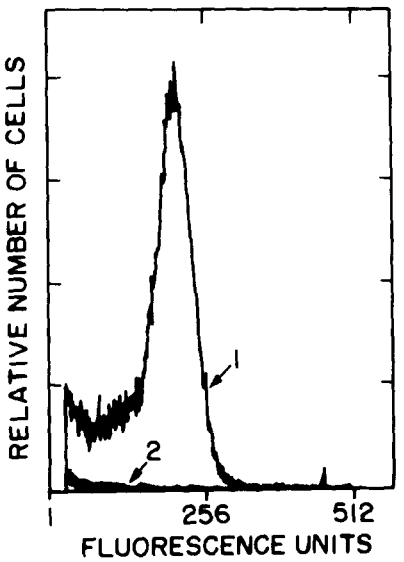

Fig. 8. Fluorescence histograms of biotinylated erythrocytes stained with microspheres-acetyl-avidin. Biotinylated erythrocytes were stained with microspheres-acetyl-avidin in the absence (1) or presence (2) of 50 μg free biotin (for details, refer to Methods).

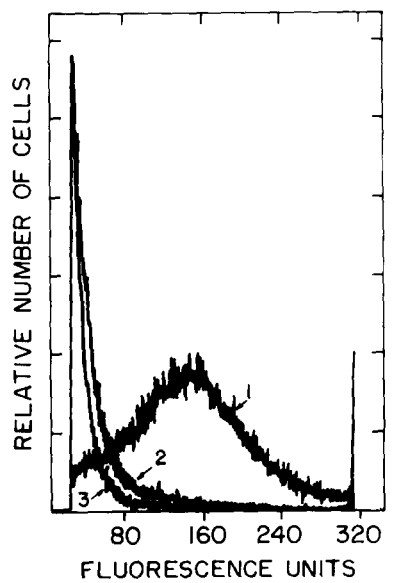

Fig. 9. Fluorescence histograms of biotinyl anti-Thy-1.2 and microspheres-acetyl-avidin labeling. $2 \cdot 10^7$ C57Bl/6J thymocytes (1 and 3) or AKR/J (2) were reacted with 0.2 ml of biotinyl anti-Thy-1.2, 8 μg/ml, 30 min at 4°C. After washing three times with phosphate-buffered saline, staining with microspheres-acetyl-avidin was performed as in Methods. In (3) binding was inhibited by 10 μg free biotin which were added to the microspheres-acetyl-avidin conjugates and pre-incubated for 10 min before their addition to cells.

Avidin binding to cell surfaces

Prior to using avidin coupled to microspheres as a universal ligand, we had to develop an avidin molecule which would bind biotin but have a low non-specific binding to cell surfaces. As a test system, human erythrocytes were biotinylated by directly coupling biotinyl hydrazide to periodate-oxidized sialic acids [10]. It was found that ^{125}I-labeled avidin [8] bound to biotinylated erythrocytes in the presence of free biotin to an extent of 11% (Table I). Increasing degrees of acetylation of the amine groups of the avidin by means of the *N*-hydroxysuccinimide ester of acetic acid resulted in a progressive decrease in the p*I*. The partially acetylated avidin (p*I* = 6.7) showed almost identical binding to that of native avidin. However, it showed negligible non-specific binding in the presence of free biotin (Table I). Increasing the acetylation beyond this point markedly reduced the specific binding although it could be shown that these highly acetylated avidins could still bind to [^{14}C]biotin (not shown).

Cells staining with microspheres-acetyl-avidin conjugates

Biotinylated erythrocytes were stained with microspheres-acetyl-avidin (p*I* = 6.7). The binding curves of ^{125}I-labeled microspheres-acetyl-avidin with and without free biotin are shown in Fig. 7. Saturation was reached at $8.6 \cdot 10^3$ microspheres per cell. The fluorescence histogram of a sample at saturation is shown in Fig. 8. Background non-specific binding in the presence of free biotin is extremely low.

In order to use the avidin-biotin complex with antibodies, we biotinylated anti-Thy-1.2 by reacting biotinyl hydrazide with periodate-oxidized antibody (see Methods). The biotinylated antibody was found to retain all of its activity (by radioimmunoassay, not shown). Thy-1.2 positive cells were reacted with microspheres-acetyl-avidin (p*I* = 6.7) in the presence or absence of free biotin. As an additional control, Thy-1.1 positive cells were also stained. From the histograms (Fig. 9), values of 5% and 10% non-specific binding were calculated for the control samples.

Discussion

Rembaum and coworkers [2,3] described the use of polymeric microspheres as carriers for ligands such as antibodies. They developed procedures to prepare such microspheres having hydroxyl and carboxyl groups at their surface. Those microspheres were mainly used for visualization of receptors in the electron microscope. For their use as fluorescent markers, fluorescein-conjugated antibodies were attached to their surface. While this work was in progress, Margel and colleagues [11, 12] developed polyglutaraldehyde microspheres containing fluorescent monomers during the synthesis. These studies established that the method is of general value. However, each of the microsphere types described had some or all of the following limitations: (1) Non-specific binding was relatively high [13]. (2) Microspheres were not stable in suspension in isotonic saline. (3) The fluorescence per sphere was limited by the number of available reactive groups in the polymer. In most cases, the fluorescent molecule and the ligand competed for the same reactive groups.

In view of the fact that high fluorescein concentrations lead to quenching of the fluorescence, we decided to incorporate the fluorophore as part of the polymer cross-bridges. Thus, a polymer with relatively distant but still sufficiently high concentration of fluorophores could be achieved. About 700 fluorophore molecules per a 500 Å-diameter microsphere could be readily introduced. Introduction of the fluorophore as a water insoluble molecule ensured its presence within the polymer matrix. Any water soluble molecule would be incorporated near the polymer surface and thus might lead to changes in the surface properties. The methacrylic carboxyls and glycine groups made the polymer non-interactive with negatively charged cell surfaces. The ethylenediamine together with the glutaraldehyde served as spacers to keep the attached ligand away from the matrix. The use of cyanoborohydride to reduce the Schiff bases formed with an amino-containing ligand stabilizes the bond at neutral pH.

Our procedure yielded mono-dispersal microspheres that form a stable suspension in isotonic solutions. A maximum of ten molecules of immunoglobulin were attached per sphere, similar to values obtained for other microspheres [2].

The analysis in the cell sorter showed that this method resulted in a highly specific determination of surface groups. In the direct and indirect labeling procedures non-specific binding was about 1%. It was calculated by means of ^{125}I-labeled microspheres-ligand binding with either antibody or acetyl-avidin that some 10^4 spheres bind per thymocyte or erythrocyte at saturation.

A comparison of the absolute fluorescence intensity of indirect immunofluorescence with microspheres indirect labeling was performed. Thy-1.2 positive cells were stained with mouse anti-Thy-1.2 and then with either microspheres-goat-anti-mouse-immunoglobulin or fluorescein isothiocyanate-goat-anti-mouse immunoglobulin. Nearly equivalent fluorescence intensities were obtained. Binding assays with ^{125}I-labeled goat-anti-mouse Fab under similar conditions gave a value of $8 \cdot 10^5$ receptors per cell [14]. Since the fluorescein isothiocyanate-goat-anti-mouse immunoglobulin contained four fluorescein molecules per protein molecule, $3 \cdot 10^6$ fluorophores were bound per cell at saturation (assuming a 1 : 1 ratio of second antibody to receptor). When microspheres-antibody staining was performed, 10^4 microspheres bound per cell, each having 700 fluorophores. Thus, $7 \cdot 10^6$ fluorophores are bound per cell. When a receptor is abundant (more than 10^5 per cell), both methods give comparable fluorescence. However, in a case where 10^3 receptors are present per cell, the use of microspheres should result in a 100-fold stronger fluorescence signal. The microspheres staining procedure is especially useful for the range below 10^4 receptors per cell. For very low numbers of receptors per cell, larger spheres could be more useful. A sphere of 1000 Å diameter, prepared similarly, would contain 8-fold higher fluorescence than the 500 Å diameter sphere. Using 0.782 μm diameter spheres, Parks et al. [15], showed that as few as some tens of microsphere-antigen conjugates per cell could be used to select hybridomas from mixtures.

Attempts were made to enhance the specificity and create a universal ligand by means of the avidin-biotin high affinity complex (10^{15} M^{-1})[16]. It was possible to prepare a partially acetylated avidin ($pI = 6.7$) whose non-specific interaction with biotinylated erythrocytes was 1.4%. When

this acetyl-avidin was coupled to microspheres, the nonspecific interaction with lymphocytes was 5–10%. As our results show, high acetylation results in a dramatic decrease in the binding capacity to biotinylated erythrocytes. This is probably due to repulsion of the negative protein from the cell surface since this acetyl-avidin retains its biotin-binding capacity (data not shown). It seems likely that the acetyl-avidin used ($pI = 6.7$), which was optimized to be non-reactive with erythrocytes, might not have had sufficient negative charge to be non-interactive with lymphocytes.

Finn, et al. [17] reported reduction in the nonspecific binding of native avidin to cells by a factor of ten upon succinylation. However, high relative non-specific binding still existed; probably due to decreased specific binding.

Biotinylation of the antibody through aldehydes formed by periodate oxidation of the hinge carbohydrate was found to be a mild procedure which preserved the antibody activity.

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