NEW IMMUNOLATEX SPHERES: VISUAL MARKERS OF ANTIGENS ON LYMPHOCYTES FOR SCANNING ELECTRON MICROSCOPY

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ABSTRACT

New immunochemical reagents consisting of antibodies bound to small latex spheres were used as visual markers for the detection and localization of cell surface antigens by scanning electron microscopy. Cross-linked latex spheres of various sizes from 300 to 3,400 Å in diameter were synthesized by aqueous emulsion copolymerization of methacrylate derivatives containing hydroxyl and carboxyl functional groups. Proteins and other molecules containing primary amino groups were covalently bonded to the acrylic spheres under a variety of mild conditions by the aqueous carbodiimide, cyanogen bromide, and glutaraldehyde methods. For use in the indirect immunochemical-labeling technique, goat antibodies directed against rabbit immunoglobulins were bonded to the spheres. These immunolatex reagents were shown to bind only to cells (red blood and lymphocytes) which had previously been sensitized with rabbit antibodies against cell surface antigens. Mouse spleen lymphocytes with exposed immunoglobulins on their surface (B cells) were labeled with these spheres and distinguished from unlabeled or T lymphocytes by scanning electron microscopy. The distribution of Ig receptors on lymphocytes was also studied using the spheres as visual markers. When lymphocytes were fixed with glutaraldehyde and subsequently labeled with the immunolatex reagents, a random distribution was observed by scanning electron microscopy; a patchy distribution was observed when unfixed lymphocytes were used. These results are consistent with studies using ferritin-labeled antibodies (S. De Petris and M. Raff. 1973. *Nature* [*Lond.*]. **241**:257.) and support the view that Ig receptors on lymphocytes undergo translational diffusion. In addition to serving as visual markers for scanning electron microscopy, these latex spheres tagged with fluorescent or radioactive molecules have applications as highly sensitive markers for fluorescent microscopy and as reagents for quantitative studies of cell surface antigens and other receptors.

Polymeric solid supports to which molecules of biochemical interest are bound have been widely used as immunoadsorbents for antigen and antibody purification (18). More recently, such immunoadsorbents have been utilized in the separation of specific populations of lymphoid cells (13,

29). Antibodies adsorbed primarily to polystyrene latex particles also have found applications in immunoassays based on the agglutination technique (10, 16). However, to date, little work has been reported on the use of very small polymeric particles as microscopic markers for antigens or antibodies on the surface of cells. Previous immunochemical-labeling methods relied on antibodies tagged with fluorescein or other dyes for the detection of antigens by fluorescent microscopy (22, 24), radioactive isotopes for use with autoradiographic techniques (30), or biological macromolecules such as ferritin (25, 31), hemocyanin (12), and viruses (1) for visualization by transmission electron microscopy. The application of latex particles for the detection and localization of cell surface molecules by scanning electron microscopy constitutes a relatively new approach (20).

Polystyrene latex particles, 0.2 μm in diameter, have recently been used as immunochemical markers for scanning electron microscopy (15). But applications of such a reagent are limited because the hydrophobic surface of the polystyrene particles makes them stick nonspecifically to many surfaces and molecules. Furthermore, reliance on weak adsorption forces to hold the antibodies on the particles is not always satisfactory (19) and chemical bonding of antibodies to polystyrene particles under mild conditions is not a well-established procedure. We have developed a new, widely applicable technique and have shown in a previous communication that it can be used to label red blood cells (RBC)[1] (20). In the present paper we report in detail: (*a*) The design and synthesis of spherical particles containing hydroxyl and carboxyl groups on their surface in a wide range of sizes (300–3,400 Å in diameter) by emulsion copolymerization. (*b*) The experimental conditions for the covalent bonding of radioactive amino acids, fluorescent molecules, and antibodies to the spheres by means of the cyanogen bromide, carbodiimide, and glutaraldehyde methods. (*c*) The successful application of antibody-tagged particles as markers for antigens on the surface of RBC and lymphocytes. (*d*) The identification of mouse spleen lymphocytes bearing Ig receptors on their cell surface (B lymphocytes) by scanning electron microscopy using immunolatex spheres as specific markers. (*e*) The phenomenon of patching of surface immunoglobulins labeled with immunolatex spheres as visualized by scanning electron microscopy.

[1] *Abbreviations used in this paper:* AP, ammonium persulfate; BSA, bovine serum albumin; EDC, 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide; EGD, ethylene glycol dimethacrylate; HEMA, 2-hydroxyethyl methacrylate; MAA, methacrylic acid; MMA, methyl methacrylate; PBS, phosphate-buffered saline; RBC, red blood cells; SDS, sodium dodecyl sulfate.

MATERIALS AND METHODS

Materials

The following monomers were obtained from Rohm and Haas Co., Philadelphia, Pa. and purified as indicated: 2-hydroxyethyl methacrylate (HEMA) distilled in the presence of 0.5% hydroquinone at 95°C, 1 mm Hg pressure; methyl methacrylate (MMA) distilled at 63°C, 200 mm Hg pressure; methacrylic acid (MAA) distilled at 60°C, 10 mm Hg pressure, and ethylene glycol dimethacrylate (EGD) distilled at 98°C, 4 mm Hg pressure.

Sodium dodecyl sulfate (SDS) was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis.; cyanogen bromide from MC&B Manufacturing Chemists, Norwood, Ohio; 25% glutaraldehyde from Polysciences Inc., Warrington, Pa.; 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) from Ott Chemical Co., Muskegon, Mich. Rabbit IgG and goat antirabbit antiserum were obtained from Pentex Biochemical, Kankakee, Ill.; rabbit antihuman red blood cell, rabbit antimouse lymphocyte, and rabbit antimouse gamma globulin antisera were obtained from Cappel.

Phosphate-buffered saline (PBS) is composed of 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, and 1.15 g Na_2HPO_4 per liter of distilled H_2O and adjusted to pH 7.40.

Methods

Synthesis of Latices

Cross-linked latices were prepared in tumbling containers at 98°C in presence of argon gas, by copolymerization of water-soluble and water-insoluble acrylic monomers in presence of an emulsifier and a cross-linking agent. The following components were introduced into distilled water: HEMA, MMA, MAA, EGD, SDS, and ammonium persulfate (AP). The reaction time was 1 h and the yields were nearly quantitative (99–100%). (See Results for conditions of synthesis of latices of different sizes.)

Emulsifier and other ionic impurities were removed from the latex suspension by chromatography on a mixed-bed ion exchange column consisting of Bio-Rad AG 1X10 and AG 50WX12 resins (Bio-Rad Laboratories, Richmond, Calif.).

The concentration of latex particles in solution was based on dry weight analysis. A known volume of solution was dried at 107°C to constant weight.

The density of the latex particles was determined by

centrifugation at 100,000 *g* for 12 h on a linear sucrose gradient. The carboxyl content of the copolymer latex was determined by potentiometric titration.

Preparation of Fluorescent or Radioactive Latex Spheres

Tritiated glycine and dansyl-ε-lysine were coupled to copolymer latex spheres (600 Å in diameter) by the cyanogen bromide procedure adapted from the method of Cuatrecasas (3). An aqueous suspension of latex spheres (20–55 mg/ml) adjusted to pH 10.5 was activated with CNBr (10–20 mg/ml of suspension) at 25°C. The pH of the reaction mixture was maintained at 10.5 with 1 N NaOH. After 10–15 min the activated spheres were added to an equal volume of 5 mM dansyl-ε-lysine or [^{3}H]glycine (1 mCi/μmol) in 0.2 M carbonate buffer at pH 10 and the suspension was stirred for 12 h at 4°C. Uncoupled reagents were removed by extensive dialysis against several changes of 0.1 M NaCl.

Preparation of Derivatized Latex Spheres

Diaminoheptane and ε-aminocaproic acid were bonded to latex spheres using the aqueous carbodiimide reaction (9). 10 mg of EDC were added with stirring to 5 ml of latex (25 mg/ml) suspended in 0.01 M diaminoheptane or 0.01 M ε-aminocaproic acid at pH 6–7 and 4°C. After stirring for 2 h in the cold, the suspension was exhaustively dialyzed against 0.1 M NaCl.

Preparation of Antibody-Latex Conjugates

For use in cell surface-labeling experiments, purified goat antirabbit IgG antibodies were covalently bonded to latex spheres by either the carbodiimide or glutaraldehyde method. In the carbodiimide reaction, 10 mg of EDC were added to 50 mg of ε-aminocaproic acid derivatized latex and 1 mg of antibody in 2 ml of 0.1 M NaCl at pH 7.0 and 4°C. After 2 h the coupling reaction was stopped by the addition of 0.2 ml of 0.1 M glycine solution pH 8.0. The glutaraldehyde coupling reaction was carried out in two steps (21). An aqueous glutaraldehyde solution was added slowly to a suspension of diaminoheptane derivatized latex in 0.01 M sodium phosphate buffer pH 7.0 to give a final concentration of 1.25% glutaraldehyde. After stirring for 1 h at 25°C, excess glutaraldehyde was removed by overnight dialysis of the latex suspension against 0.1 M NaCl-0.01 M phosphate buffer pH 7.0 at 4°C. Goat antirabbit IgG (1–2 mg) was added to 50 mg of activated latex spheres in 5 ml of 0.01 phosphate buffer at pH 7.0 and the suspension was stirred for 5 h at 25°C. The antibody-latex conjugate was separated from the uncoupled antibody as follows: The reaction suspension was layered onto a gradient consisting of a 58% (wt/wt) sucrose solution overlayered with 10% sucrose solution buffered at pH 8.0 with 0.01 M glycine. After centrifugation at 100,000 *g* for 3 h in a Beckman SW-27 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), the latex conjugate was collected at the interface between 58 and 10% sucrose solutions. This procedure was repeated to insure complete removal of unbound antibody. Finally, the antibody-latex conjugate was dialyzed extensively against PBS at pH 7.4. Large aggregates were removed by centrifugation at 4,000 *g* for 10 min and the conjugate (15–20 mg/ml) was stored at 4°C.

Purification and Radioactive Labeling of Antibodies

Goat antirabbit IgG was purified from whole serum on an immunoadsorbent consisting of rabbit IgG bound to Sepharose 4B by the CNBr method (3). The adsorbed antibodies were eluted with 3 M sodium thiocyanate (7). Rabbit IgG and purified goat antirabbit IgG were labeled with carrier-free Na^{125}I using lactoperoxidase bound to Sepharose 4B according to the procedure of David (5). Protein concentrations were determined from optical density measurements using an extinction coefficient ($\epsilon_1^{1\ \frac{mg}{ml}}$) at 280 nm of 1.4.

Quantitative Measurements of Bonding to Latex Spheres

Covalent bonding of [^{3}H]glycine and [^{125}I]rabbit IgG was carried out in duplicate on a microscale under similar conditions as described above. In a typical measurement, the carbodiimide coupling reaction was initiated by the addition of 5 μl of EDC (40 mg/ml) to a test tube containing 50 μl of latex spheres (27 mg/ml) suspended in 0.011 M [^{3}H]glycine ($1–2 \times 10^3$ cpm/nmol) or 0.1–1 mg/ml [^{125}I]rabbit IgG (1×10^6 cpm/nmol) in 0.1 M NaCl pH 7.0 at 4°C. A final concentration of 1.25% glutaraldehyde was used in the coupling of rabbit IgG to diaminoheptane derivatized latex spheres in a one-step procedure. The reaction was terminated by the addition of glycine buffer. Unbound [^{3}H]glycine was separated from the tritiated latex by aggregation of the latex with 2 ml of 0.1 M glycine buffer pH 2.8, centrifugation at 1,500 *g* for 15 min, and resuspension in 0.1 ml of 0.1 M borate buffer pH 8.0. This procedure was repeated four times. The final pellet was resuspended in a given volume of borate buffer and an aliquot was dissolved in Aquasol (New England Nuclear, Boston, Mass.) for radioactivity counting. Latex labeled with [^{125}I]rabbit IgG was separated from unbound IgG by dilution with 0.1 M glycine buffer pH 8.0 followed by repeated centrifugation at 100,000 *g* for 30 min.

Preparation and Labeling of Cells

RBC: Human RBC (type O) were washed two times with PBS containing 0.3% dextrose. 0.5 ml of rabbit antihuman RBC antiserum diluted 1:1 in PBS was added to 0.5 ml of RBC (4×10^7 cells). After incubating for 15 min at 25°C, the cells were washed in PBS containing 0.2% bovine serum albumin (PBS-BSA) by repeated centrifugation at 400 *g* for 10 min. To 0.1 ml of sensitized

RBC (5×10^6 cells) was added 0.1 ml of goat anti-rabbit-latex conjugate. After 20 min at 25°C, the cells were washed four times in PBS by centrifugation at 400 *g*.

MOUSE SPLEEN LYMPHOCYTES: Cell suspensions were obtained by gently teasing spleen tissues from female Balb/c mice into PBS. The lymphocytes were separated from RBC and dead cells by centrifugation over a Ficoll-Isopaque solution (2) and subsequently were washed twice in PBS-BSA. A viability of greater than 85% by means of trypan blue was observed.

Labeling studies were carried out on cells either sedimented onto glass cover slips or maintained in suspension. In the former procedure, $1-3 \times 10^6$ cells were centrifuged at 150 *g* for 15 min onto glass cover slips (12-mm diameter) placed in Satorius filter holders (SM 165 14, Satorius Balances, sold by Brinkmann Instruments, Inc., Westbury, N. Y.). The cover slips coated with cells were carefully removed so as to prevent drying and rinsed in PBS-BSA. In some experiments sodium azide (5 mM) was introduced into the buffers to inhibit capping. The cells were then incubated at 25°C for 15 min or at 4°C for 60 min with rabbit antiserum against either mouse lymphocytes or mouse γ-globulin which had been diluted with 1 vol of PBS. After washing by repeatedly dipping the cover slips into buffer, the sensitized cells were labeled with goat antirabbit IgG-latex conjugate (14 mg/ml) at 25°C for 20 min or at 4°C for 60 min and rinsed in PBS. Lymphocytes in suspension were labeled by a procedure similar to that described for RBC.

In some experiments, lymphocytes were prefixed in 0.25% glutaraldehyde-PBS for 20 min at 25°C and subsequently incubated in PBS containing 0.01 M ε-aminocaproic acid for 30 min before labeling. In control experiments nonspecific rabbit antiserum was substituted for the lymphocyte-specific rabbit antiserum.

Preparation of Samples for Scanning Electron Microscopy

For scanning electron microscopy, cells were centrifuged onto glass cover slips as described above. The adhesion of glutaraldehyde-fixed cells onto glass cover slips was significantly increased by pretreating the cover slips with a solution of 3,3-ionene chloride (4 μg/ml), a positively charged synthetic polymer (27), followed by rinsing with PBS. The ionene forms a monolayer on the cover slip.

Samples were fixed with freshly prepared 1.25% glutaraldehyde solution in PBS for 1 h at 25°C, rinsed in PBS buffer, and postfixed in 1% osmium tetroxide-0.1 M collidine buffer pH 7.4 for 1 h. Dehydration was carried out in a series of ethanol solutions from 50 through 95% for 5 min each and finally in three changes of 100% ethanol over a period of 3 h. After critical point drying from Freon 13 (17), the samples were coated with gold on a rotatory platform. Cells were examined with an ETEC scanning electron microscope operated at 20 kV.

RESULTS

Synthesis of Latex Spheres by Emulsion Copolymerization

The main components of the aqueous emulsion polymerization system were two water-soluble monomers (HEMA and MAA) and a water-insoluble monomer (MMA) resulting in a high concentration of hydroxyl and carboxyl groups on the surface of the spheres. The actual composition of the polymerizing mixture, the yields, and the diameters of particles determined by scanning electron microscopy are shown in Table I. The diameter of these particles measured by transmission electron microscopy is approximately 200 Å smaller than by scanning electron microscopy presumably due to the gold coating used in the latter. It can be approximately calculated that particles with a diameter of 600 Å have 4,200 carboxyl groups per particle (based on potentiometric titration and a density of 1.24 g/cm^3).

The uniform nature of the methacrylate spheres synthesized by aqueous emulsion copolymerization is illustrated in Fig. 1.

Bonding of [^3H]Glycine

Studies of the covalent bonding of tritiated glycine to copolymer acrylic latex spheres (600 Å in diameter) by the aqueous carbodiimide and cyanogen bromide methods are summarized in Table II. Under the reaction conditions listed, the latex particles remained largely as a monodispersed suspension. However, when the latex suspension was exposed to pH values below 5.5 where the carboxyl groups titrate or when the carbodiimide reaction was carried out at high EDC concentrations (>20 mg/ml), for long reaction times (>3 h) or at room temperature, aggregation of the particles was observed. By the carbodiimide procedure the extent of bonding of glycine to the spheres was strongly dependent on the glycine concentration over the range 1–10 mM, but less dependent on either the EDC concentration (2–20 mg) or the pH (6–7) of the reaction. HEPES buffer had no significant effect. By the cyanogen bromide procedure, a greater number of glycine molecules could be bound to the spheres when the coupling reaction was carried out at basic pH. At pH 10, the

TABLE I

Emulsion Copolymerization of Methacrylates

Run	Percent concentration (wt/wt) HEMA	MMA	MAA	EGD	Total monomer	SDS	AP	Diameter*	Percent of solid‡
								Å	
1	0.9	1.71	0.3	0.09	3.0	0.120	0.013	300	No solid
2	2.0	3.82	0.66	0.20	6.49	0.110	0.012	600 ± 90	No solid
3	2.1	3.99	0.7	0.21	7.0	0.110	0.012	750 ± 100	No solid
4	3.3	6.27	1.1	0.33	11.0	0.108	0.011	—	No solid
5	4.5	8.55	1.5	0.45	15.0	0.097	0.011	1,400 ± 110	No solid
6	4.8	9.12	1.6	0.48	16.0	0.097	0.010	1,550 ± 120	No solid
7	7.5	14.25	2.5	0.75	25.0	0.092	0.010	2,300 ± 170	3.2
8	9.0	17.1	3.0	0.90	30.0	0.086	0.009	2,900 ± 150	5.0
9	10.5	19.9	3.5	1.05	35.0	0.079	0.008	3,400 ± 120	6.5

* Determined by scanning electron microscopy.

‡ Percent solid remaining after filtration of the latex through Whatman no. 1 filter paper.

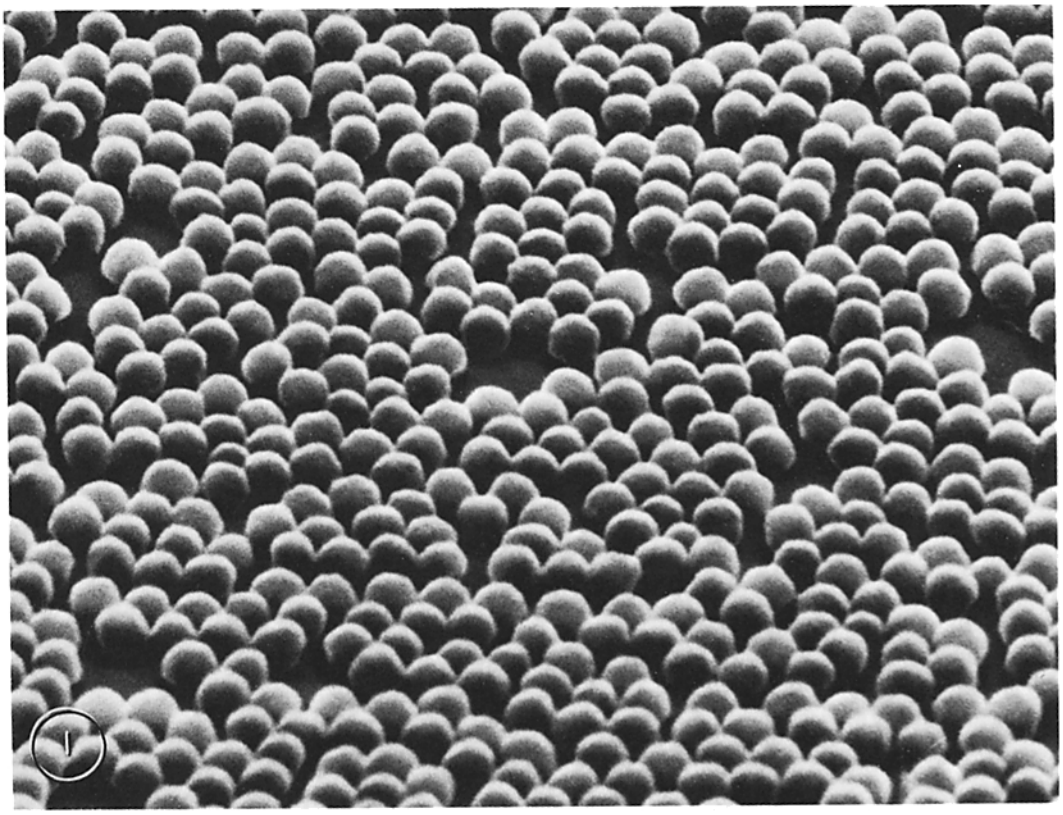

FIGURE 1 Scanning electron micrograph of copolymer latex spheres (1,400 Å in diameter) synthesized by aqueous emulsion copolymerization.

TABLE II

Bonding of [^{3}H]Glycine to Latex Spheres at 4°C

Glycine concn	EDC concn	Time	Buffer	pH	[^{3}H]Glycine incorporation	
mol/liter	*mg/ml*	*h*	*mol/liter*		*nmol/mg*	*mol/mol**
Carbodiimide reaction						
0.01	2.0	1	—	6.0	0.330	27.5
0.01	2.0	3	—	6.0	0.550	45.8
0.001	2.0	1	—	6.0	0.036	3.0
0.01	20.0	1	—	6.0	0.775	64.5
0.01	2.0	1	0.1 HEPES	6.0	0.315	26.2
0.01	2.0	1	0.1 HEPES	7.0	0.150	12.5
0.01	—	3		6.0	0.008	0.67
CNBr reaction‡						
0.01		1	0.1 M carbonate	10.0	5.920	493.0
0.01		4	0.1 M carbonate	10.0	8.700	725.0
0.01		8	0.1 M carbonate	10.0	9.520	795.0
0.01		1	0.1 M phosphate	7.0	0.232	19.3
0.01		4	0.1 M phosphate	7.0	0.527	44.0
0.01		8	0.1 M phosphate	7.0	0.850	70.7

* Moles of [^{3}H]glycine incorporated per mole of latex sphere is based on an average diameter of 600 Å and a density of 1.24.

‡ Latex spheres activated for 10 min with 10 mg/ml of CNBr at 25°C and pH 10.5. Coupling reaction was subsequently carried out at 4°C.

cyanogen bromide reaction was about 90% complete after 4 h at 4°C, and for a glycine concentration of 10 mM over 700 molecules of glycine were bound per latex sphere.

Bonding of [^{125} I]Rabbit IgG

The effect of rabbit IgG concentration on the bonding of [^{125} I]rabbit IgG to copolymer acrylic latex spheres by the cyanogen bromide, carbodiimide, and glutaraldehyde reactions is shown in Fig. 2. A linear dependence was observed when these reactions were carried out at pH 7 for 1 h at 4°C. Under these conditions glutaraldehyde coupling of rabbit IgG to spheres previously derivatized with diaminoheptane was most effective and on the basis of a sphere diameter of 600 Å and a density of 1.24 mg/ml, over eight antibody molecules were bound per latex sphere at rabbit IgG and glutaraldehyde concentrations of 1 mg/ml and 1.25%, respectively. The low amount of protein bound by the CNBr method was due in part to the low efficiency of this reaction at neutral pH (Table II). As in the case of [^{3}H]glycine, binding of rabbit IgG in the absence of coupling reagent was low.

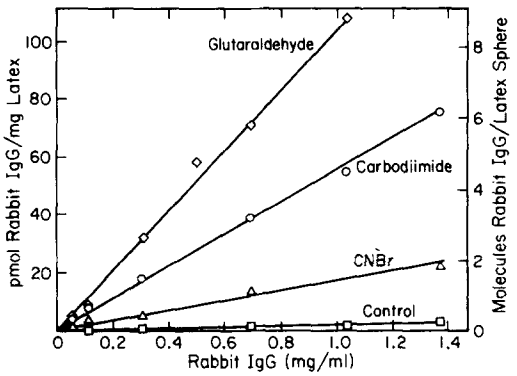

FIGURE 2 Effect of rabbit IgG concentration on the covalent bonding of [^{125} I]rabbit IgG to latex spheres 600 Å in diameter. Reactions were carried out at 4°C for 1 h. In the CNBr reaction the latex spheres were initially activated with 20 mg/ml CNBr at pH 10.5 and subsequently coupled to rabbit IgG in 0.1 M phosphate buffer at pH 7.0. The carbodiimide reaction was carried out at pH 7.0 in the absence of added buffer using a final EDC concentration of 2 mg/ml. In the glutaraldehyde reaction rabbit IgG was coupled to diaminoheptane-derivatized latex spheres in 0.1 M phosphate buffer at pH 7.0 in the presence of 1.25% glutaraldehyde. The control experiment was carried out in the absence of coupling reagents.

Antibody-Latex Conjugates

The presence of hydroxyl and carboxyl functional groups on the latex spheres enabled antibodies and other molecules containing primary amino groups to be covalently bonded to the spheres by any of the chemical procedures mentioned above. In applications as immunochemical reagents for fluorescent and electron microscopy and for quantitative studies, antibody-latex conjugates were prepared according to the scheme in Fig. 3. Copolymer latex spheres (600 Å in diameter) activated with cyanogen bromide were labeled with either tritiated glycine or dansyl-ϵ-lysine under conditions which yield a high degree of labeling (Table II). The tagged spheres were subsequently derivatized with either ϵ-aminocaproic acid or diaminoheptane using the carbodiimide method. Such "spacer" molecules which extend functional groups used in protein coupling reactions from the insolubilized matrix have been found useful in the purification of proteins by affinity chromatography (4).

Finally, antibody molecules were conjugated to underivatized or ϵ-aminocaproic acid-derivatized latex spheres by the carbodiimide reaction or to diaminoheptane-derivatized spheres by the glutaraldehyde reaction. Goat antirabbit IgG-latex conjugates prepared by either coupling procedure were shown to be immunochemically active as measured by binding to cells sensitized with specific rabbit antigens. However, higher activity was generally obtained with conjugates prepared by a two-step glutaraldehyde method in which the diaminoheptane-derivatized latex spheres were first activated with an aqueous solution of glutaraldehyde and subsequently coupled to goat antirabbit IgG after removal of the excess coupling reagent. This two-step reaction, which has been used recently to prepare highly active ferritin-antibody conjugates (21), has the advantage of efficiently coupling antibodies to the latex spheres under mild conditions which avoid intra- and intermolecular cross-linking of antibody molecules. Latex conjugates prepared by this procedure showed no loss in activity over 3 mo when stored at 4°C in the presence of 10^{-4} M NaN_3.

Latex spheres, 1,400 Å in diameter, were shown to have applications in immunological assays. Copolymer latex particles bonded to rabbit IgG by either the cyanogen bromide or carbodiimide reactions agglutinate in the presence of serial dilutions of goat antirabbit IgG antiserum.

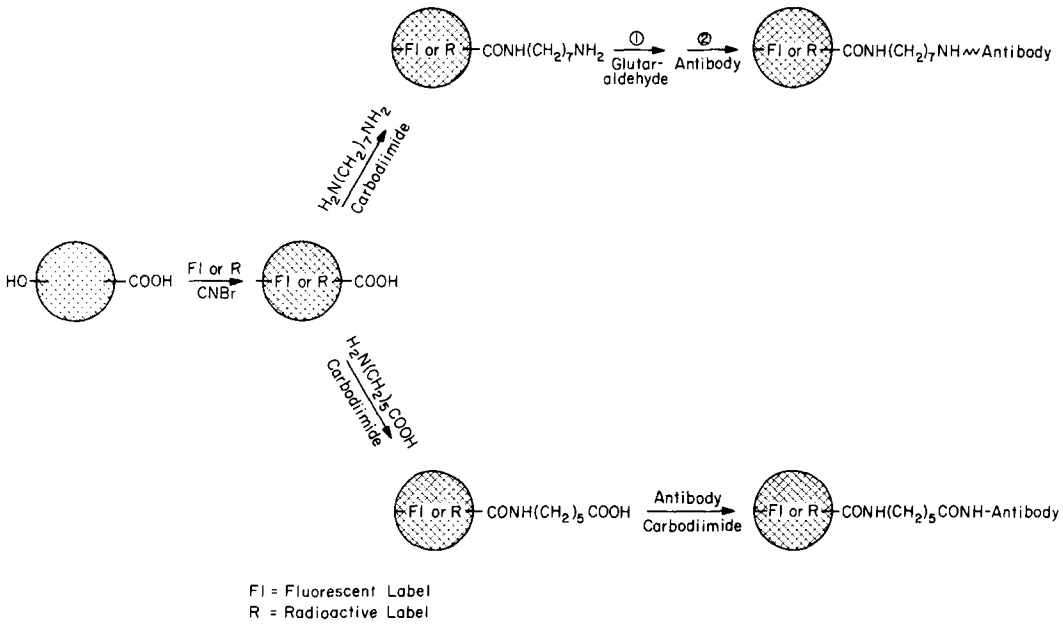

FIGURE 3 Scheme depicting the sequence of reactions used to prepare radioactive- and fluorescent-labeled latex-antibody conjugates for cell surface-labeling studies.

Labeling of Cells with Immunolatex Spheres

The application of latex spheres as quantitative reagents and visual markers for cell surface antigens was demonstrated on RBC and lymphocytes using the indirect immunochemical technique. Immunolatex conjugates consisting of an average of one to two molecules of goat antirabbit IgG per latex sphere of 600 Å diameter were used in these labeling studies. [^{125}I]Goat antirabbit IgG antibodies were used to measure the number of molecules bound per latex sphere.

RBC: The binding of [^{3}H]glycine-labeled immunolatex particles to human RBC sensitized with heterologous rabbit antihuman RBC antibodies is shown in Fig. 4. At goat antirabbit IgG-latex concentrations greater than 3 mg/ml, a saturating value of 3.5 ± 0.5 × 10^4 conjugates per cell is reached (calculated from Fig. 4). It can be calculated that a maximum of 4.7 × 10^4 spheres of diameter 600 Å can be distributed in a monolayer on a RBC of surface area 145 μm^2 (32). Scanning electron micrographs of RBC or lymphocytes (Fig. 6 *a*) labeled with particles at saturating goat antirabbit IgG-latex concentrations confirm the tight packing of the spheres on the cell surface. Scanning electron micrographs of RBC tagged with immunolatex spheres 800 and 1,300 Å in diameter have previously been presented (20); RBC tagged with immunolatex spheres 300 Å in diameter indicate that spheres of this size can also serve as visual markers for scanning electron microscopy. In the control experiments, nonspecific binding of goat antirabbit IgG-latex conjugates to unsensitized RBC was generally quite low as determined either by radioactivity measurements (Fig. 4) or scanning electron microscope visualization (20).

LYMPHOCYTES: Mouse spleen lymphocytes fixed in suspension, sedimented onto ionene-treated glass cover slips, and prepared for scanning electron microscopy by the critical point drying procedure exhibited many of the features described for human peripheral blood lymphocytes (23). Cells were spherical in shape with a diameter of 4.1–5.8 μm and surface morphology ranging from smooth, without surface projections to highly complex with microvilli covering much of the cell surface. Most of the lymphocytes, however, displayed smooth surfaces with a variable number of either short stublike projections or longer microvilli of average length 0.6 μm randomly distributed over the cell surface (Fig. 5 *a*). When the lymphocytes were incubated in buffer containing 5 mM NaN_3 for over 1 h, many of the cells were found to exhibit elongated microvilli-like structures (Fig. 5 *b*).

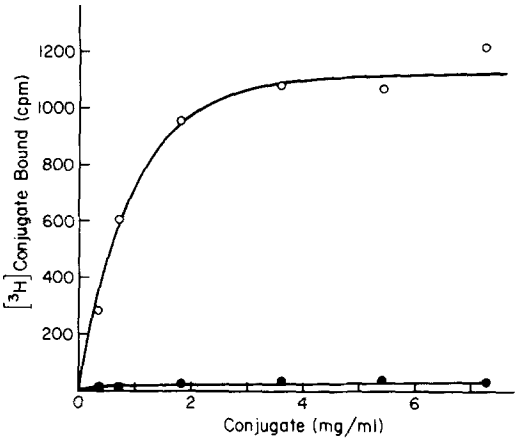

FIGURE 4 The effect of goat antirabbit IgG-latex conjugate concentration on the binding of tritiated conjugates to 2.2 × 10^6 RBC sensitized with rabbit anti-RBC antibodies. In the control experiment unsensitized RBC were used to measure nonspecific binding of the conjugates.

The labeling of mouse spleen lymphocytes with immunolatex particles was tested with cells treated with rabbit antimouse lymphocyte antiserum in the presence of NaN_3 to inhibit capping (26). As shown in Fig. 6 *a*, latex spheres cover the cell surface in a tightly packed array and line the microvilli. Lymphocytes with and without microvilli were heavily labeled with the spheres.

In the control experiment in which nonspecific rabbit antiserum was substituted for antimouse lymphocyte antiserum, little, if any, binding of goat antirabbit IgG-latex conjugates to the lymphocytes was observed (Fig. 6 *b*). When the labeling was carried out on cells layered onto glass cover slips, however, a high degree of conjugate binding to the glass surface was observed in both the control and the specifically labeled samples (Figs. 6 *a* and *b*).

Lymphocytes which have immunoglobulins on their cell surface were identified by first treating the cells with rabbit antimouse gamma globulin and then with goat antirabbit IgG-latex conjugates. By this method 40–50% of the mouse spleen lymphocytes were specifically labeled, as observed by fluorescent microscopy using dansyl-ε-lysine-

tagged conjugates or by scanning electron microscopy. Figs. 7 *a* and *b* represent typical scanning electron micrographs of labeled and unlabeled lymphocytes. In order to preserve the surface morphology and restrict the movement of membrane proteins, the cells were lightly fixed with glutaraldehyde before labeling. Under these conditions, latex spheres were found to be randomly distributed over the cell surface and microvilli of the lymphocytes bearing Ig receptors. The surfaces of some specifically labeled lymphocytes were more densely covered with immunolatex spheres than others. Many unlabeled lymphocytes were found with short stublike projections; some, how-

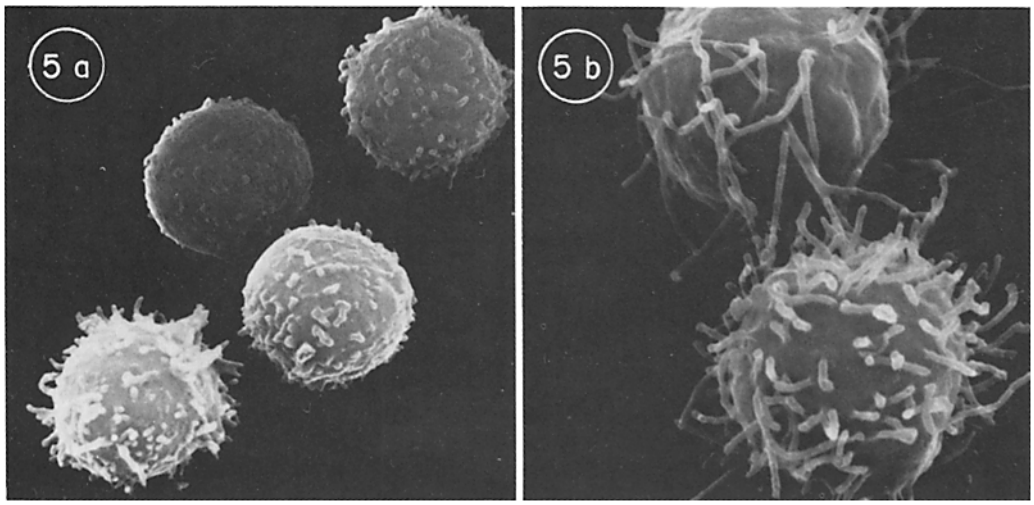

FIGURE 5 (*a*) Mouse spleen lymphocytes isolated by the Ficoll-Isopaque method, fixed in suspension with 1.25% glutaraldehyde-PBS at 25°C, and sedimented onto an ionene-treated glass cover slip. × 4,800. (*b*) Mouse spleen lymphocytes incubated for 2 h in PBS containing 5 mM sodium azide before fixation. × 7,000.

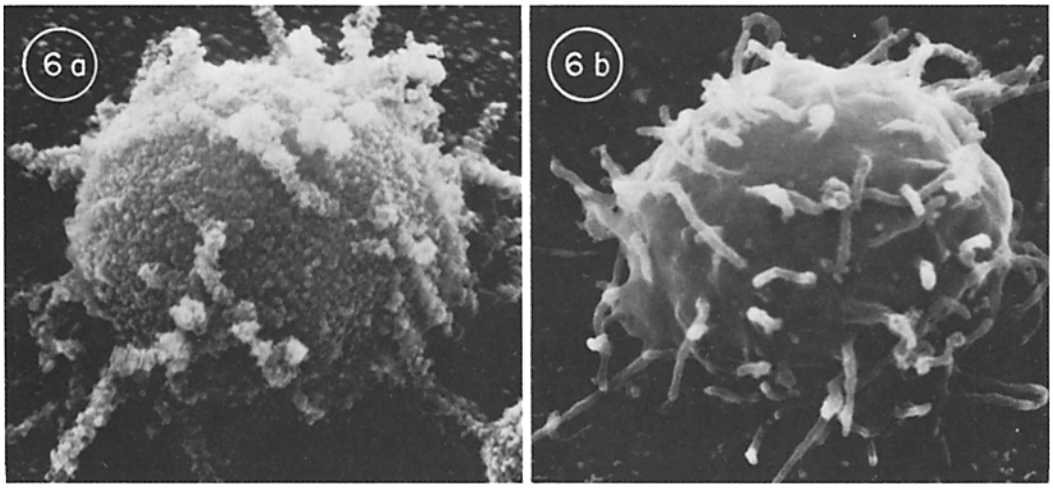

FIGURE 6 (*a*) Mouse spleen lymphocyte treated with rabbit antimouse lymphocyte antiserum followed by goat antirabbit IgG-latex conjugate. Latex markers have a diameter of about 750 Å as measured by scanning electron microscopy. Labeling was carried out in PBS containing 5 mM NaN_3 at 25°C on cells sedimented onto a glass cover slip. (*b*) Control for Fig. 6 *a* in which nonspecific rabbit serum was substituted for rabbit antimouse lymphocyte antiserum. × 11,200.

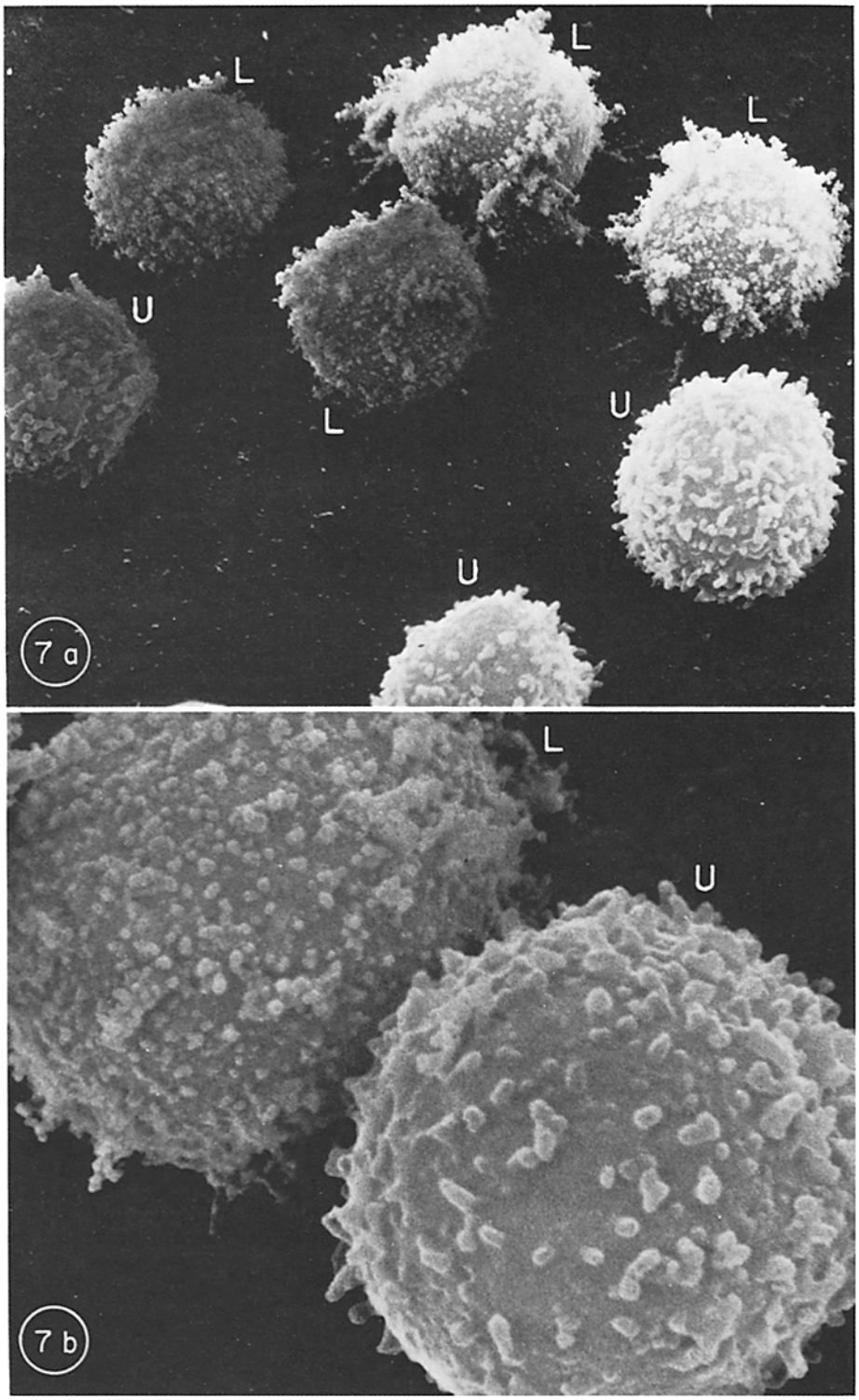

FIGURE 7 (*a*) Mouse spleen lymphocytes fixed at 25°C with 0.25% glutaraldehyde-PBS for 20 min and then treated with rabbit antimouse γ-globulin antiserum followed by goat antirabbit IgG-latex conjugate. Latex markers have a diameter of about 750 Å as measured by scanning electron microscopy. Labeling was carried out with cells in suspension. Microvilli are seen on labeled (*L*) lymphocytes as well as some unlabeled (*U*) lymphocytes. × 7,700. (*b*) Lymphocytes treated as in Fig. 7 *a*. Both the labeled and unlabeled lymphocytes have short projections on their surface. Immunolatex spheres are randomly distributed on the cell surface and microvilli. × 15,600.

ever, were found with microvilli covering much of their surface.

When unfixed lymphocytes were indirectly labeled for Ig receptors at 4°C in the presence or absence of NaN_3 and subsequently fixed with glutaraldehyde also at 4°C, the immunolatex markers were distributed in large patches (Fig. 8 *a*). Under these conditions latex spheres were not present on the microvilli. Most labeled and unlabeled lymphocytes (Figs. 8 *a* and *b*) exhibited long microvilli, up to 2 μm in length, extending from their smooth cell surfaces. A similar effect of low temperature on the surface topology of cultured lymphocytes has been recently reported (14).

DISCUSSION

A new class of immunochemical reagents consisting of antibodies covalently bonded to polymeric microspheres has been prepared and shown to serve as convenient markers for the detection of cell surface antigens by scanning electron microscopy. These reagents have been used to locate antigens on RBC and Ig receptors on the surface of mouse spleen lymphocytes.

The specially designed microspheres containing HEMA and MAA were polymerized in an aqueous emulsion system so as to have hydroxyl and carboxyl groups on their surface. These hydrophilic and negatively charged groups prevent the spheres from binding nonspecifically to cell surfaces and inhibit aggregation of the spheres at neutral pH. The presence of negative charges on the surfaces of the spheres can be inferred from the fact that particle aggregation occurs over a pH range over which the carboxyl groups titrate, as well as in the presence of high concentrations of divalent cations. These functional groups were also necessary for the chemical bonding of proteins and other molecules of biochemical interest to the spheres. The microspheres were synthesized in the presence of a cross-linking agent to maintain their stability and size in both aqueous and organic solvents commonly used in the preparation of biological specimens for electron microscopy.

Studies using radioactively labeled glycine and rabbit IgG confirm that molecules containing primary amino groups can be covalently bonded to these latex spheres. The chemical bonding is achieved by activating the hydroxyl groups with cyanogen bromide or the carboxyl groups with EDC. Alternatively, a relatively small number of molecules containing free amino groups can be first bonded to the spheres. These groups can be activated with glutaraldehyde and used to couple proteins to the spheres. Antibodies bound to the latex spheres by these chemical procedures are immunologically active as determined either by the agglutination of the antibody-latex conjugates in the presence of antigens or by the specific binding of these reagents to cells sensitized with antigens as measured by radioactivity assays, fluorescent microscopy, or scanning electron microscopy.

The application of immunolatex spheres as markers for scanning electron microscopy is of particular interest in relation to the current investigations directed toward identifying T and B lymphocytes. From previous studies using fluorescent- (22, 24), radioactive- (30), and ferritin- (12, 25) labeled antibodies, it is now generally agreed that B cells have the Fab region of immunoglobulins exposed on their surface, while T cells do not (8, 11). Efforts to distinguish T from B cells on the basis of their cellular morphology by light and transmission electron microscopy, however, have been unsuccessful. Recently, Polliack et al. (23) have reported that human B lymphocytes can be distinguished from T lymphocytes by differences in their surface architecture observed by scanning electron microscopy. Lymphocytes with microvilli covering much of their surface were designated as B cells, whereas those with relatively smooth surfaces were identified as T cells. The results reported here using immunolatex spheres as specific markers for B cells, however, indicate that, at least for mouse lymphocytes, this distinction between B and T cells is not clear. Whereas many lymphocytes labeled for Ig receptors (B cells) have numerous microvilli, some do not. Likewise unlabeled cells (T cells) were found with and without microvilli. These results point out the uncertainty inherent in attempting to classify lymphocytes on the basis of surface architecture alone. Difficulties in identifying T and B lymphocytes on the basis of their surface features also may arise as a result of the existence of subpopulations of lymphocytes (11) and the sensitivity of lymphocytes to external conditions. Since it has been found that the surface topology of lymphocytes is grossly affected by temperature (14) and metabolic inhibitors such as sodium azide, it is possible that the surface features may also be sensitive to conditions used in the isolation of lymphocytes. Hence, we conclude from these studies that the identification of T and B lymphocytes by scanning electron microscopy based on morphological criteria alone is unrelia-

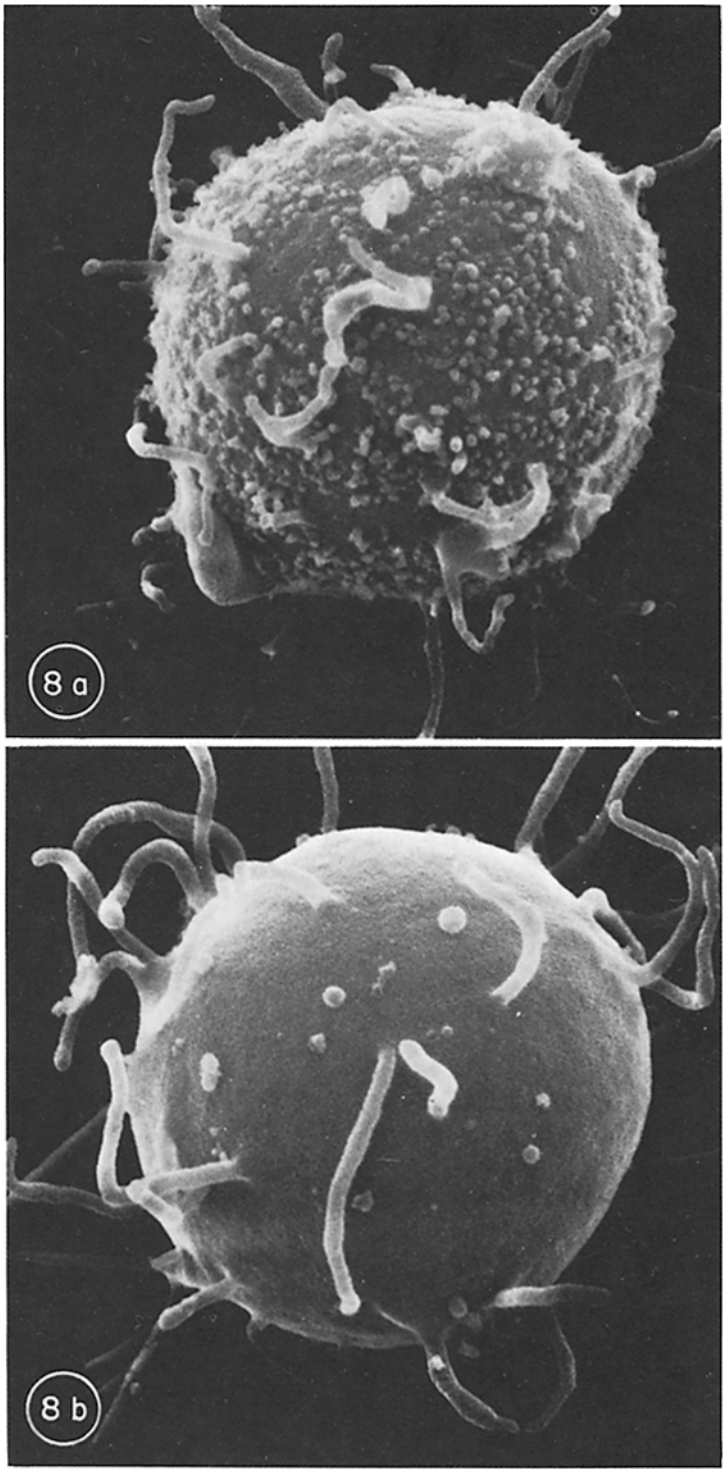

FIGURE 8 Mouse spleen lymphocytes incubated with rabbit antimouse γ-globulin and then treated with goat antirabbit IgG-latex conjugate. Latex markers have a diameter of about 750 Å as measured by scanning electron microscopy. Labeling and subsequent glutaraldehyde fixation was carried out at 4°C for 1 h with cells in suspension. × 19,600. (*a*) Lymphocyte with immunolatex spheres distributed in patches on its surface but absent on the microvilli. This is in contrast to the dense, random distribution observed when cells are fixed with glutaraldehyde before labeling as in Figs. 7 *a* and *b*. (*b*) Lymphocyte without immunolatex spheres on its surface.

ble. Identification of T and B cells by scanning electron microscopy is better achieved using immunological-labeling techniques such as described here.

The random distribution of latex spheres located on Ig receptors of *prefixed* lymphocytes is consistent with the random distribution of ferritin-conjugated monovalent Fab antimouse Ig antibodies used to detect surface Ig receptors of unfixed lymphocytes by transmission electron microscopy (6). The patchy distribution of latex particles observed when *unfixed* B lymphocytes are labeled with immunolatex spheres confirms previous results (6) with ferritin-labeled divalent antibodies. The observed patchy distribution has been postulated to be due to cross-linking of surface Ig receptors by divalent antibodies (6). The difference in latex particle distribution observed on fixed and unfixed lymphocytes is consistent with free diffusion of surface immunoglobulins in a fluid membrane (28).

In conclusion, these new reagents offer a number of advantages and applications for the study of cell surfaces, for immunodiagnosis and immunotherapy: (*a*) Latex spheres can be synthesized in a wide range of sizes and compositions to suit particular requirements and can be stored indefinitely. (*b*) Biological molecules such as antibodies, lectins, hormones, and toxins can be bound to the latex spheres by any of a variety of standard chemical procedures for use in the identification of specific populations of cells, as well as in the detection and localization of specific cell surface receptors. However, receptors which are densely distributed on the surface of cells cannot be mapped to a high resolution with these markers due to the relatively large size of the latex spheres. (*c*) Different sizes of spheres can be used in multiple-labeling experiments and in conjunction with different types of microscopy. For example, acrylic spheres the size of ferritin and hemocyanin, i.e., 150–350 Å in diameter, can serve as markers for transmission electron microscopy as well as in high resolution scanning electron microscopy; spheres larger than 0.2 μm in diameter can be used with ordinary light microscopy. (*d*) These microspheres can serve as highly sensitive fluorescent probes and quantitative reagents for biochemical and immunological studies. Binding fluorescent dyes or radioactive molecules to the microspheres instead of to the antibodies permits a high degree of tagging without adversely affecting the antibody activity.

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