A Novel Approach for Scanning Electron Microscopy of Colloidal Gold-labeled Cell Surfaces

E. DE HARVEN, R. LEUNG, and H. CHRISTENSEN
Department of Pathology, University of Toronto, Toronto, Ontario, Canada M5G 1L5

ABSTRACT   A method is described for the use of scanning electron microscopy on the surface of gold-labeled cells. It includes the use of 45- or 20-nm colloidal gold marker conjugated with Staphylococcal protein A. The marker is best recognized on the basis of its atomic number contrast by using the backscattered electron imaging mode of the scanning electron microscope. When the backscattered electron signal is mixed with the secondary electron signal, an optimum correlation between the distribution of the labeled sites and the cell surface structures is demonstrated. The method is illustrated by its application to the identification of human circulating granulocytes. Its good resolution, high contrast, and good labeling efficiency offers a promising approach to the specific localization of cell surface antigenic sites labeled with particles of colloidal gold.

When reproducible methods for the preparation of cultured cells for scanning electron microscopy (SEM)[1] were first introduced by Porter et al. (1), it became apparent that the labeling of large portions of the cell surface could benefit from the three-dimensional view offered by this instrument. As early as 1972, Lo Buglio et al. (2); and two years later, Weller (3), demonstrated that immunoscanning electron microscopy permits the specific correlation of the presence of a given antigen with the surface morphology of well-preserved cells. This was achieved with markers like latex spheres (230 nm in diameter) or haemocyanin molecules (35 × 50 nm in diameter), the sizes of which were compatible with the resolution of the SEM of conductively-coated specimens. These markers were recognized on the basis of their size and/or shape, as it appeared with the SEM in the secondary electron imaging (SEI) mode. Although it is possible to resolve ferritin molecules with higher resolution SEM (4), this marker could not find many practical SEM applications. Results obtained in several laboratories with a variety of markers in the 40–200 nm size range, which includes biological macromolecules (3), viruses (5), and co-polymer microspheres (6), were expertly reviewed by Molday et al. (7). Markers of such size are unlikely to provide high resolution surface labeling, primarily because steric hindrance phenomena prevent the labeling of many adjacent epitopes (8). In addition, large size markers obliterate too much of the underlying surface structure, mak-

ing precise topographical relationships difficult to establish.

Recently, however, it has been reported that (a) relatively stable conjugates between 5- to 40-nm colloidal gold particles and highly-purified proteins like Staphylococcus aureus protein A or antibodies can be prepared and are readily available (9, 10); (b) that colloidal gold particles can be recognized in the backscattered electron imaging (BEI) mode of the SEM (11); and, (c) finally, that the resolution of backscattered electron detectors has been improved. Taking advantage of these new developments in our current studies of leukocyte subpopulations, a novel approach has emerged for the SEM of immuno-labeled cell surfaces. This method, based on the atomic number contrast of the gold marker, is expected to be applicable to a variety of experimental cell systems and is the subject of this preliminary report.

MATERIALS AND METHODS

Collection and Separation of Peripheral Blood Leukocytes: Whole venous blood from several healthy volunteer donors was collected in citrated vacutainer tubes. The blood was rapidly transferred to the SEM laboratory at room temperature, and leukocytes were immediately separated by unit gravity sedimentation after the addition of 3% dextran (12). The collected cells were resuspended in 5 ml of RPMI-1640 medium, and the following labeling procedure was applied.

After two washes with phosphate-buffered saline, approximately 10^4 cells were attached to a 200 mesh copper grid that had been prepared with a Formvar film coated with carbon and pretreated with a 0.1% solution of poly-l-lysine hydrobromide according to Mazia et al. (13). Carefully avoiding any risk of air drying, the cells were then fixed with a 0.25% buffered glutaraldehyde solution, pH 7.2, for only 5 min, and then extensively rinsed in a 0.1% solution of glycine in phosphate-buffered saline. The cell-carrying grid was then incubated

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[1] *Abbreviations used in this paper:*   BEI, backscattered electron imaging; SEI, secondary electron imaging; SEM, scanning electron microscopy; and TEM, transmission electron microscopy.

with an appropriate dilution of the monoclonal antibody D2,[2] supplemented with 1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) at room temperature for 30 min. The monoclonal antibody D2 being of the IgM subclass, a second incubation followed, with a goat anti-mouse IgM, also for 30 min. Finally, a third incubation followed with a protein A/colloidal gold complex prepared according to Horisberger (14) and DeMey (10). The colloidal gold particles, obtained by reducing chloroauric acid with sodium citrate according to Frens (15), were prepared in two sizes, 45 and 20 nm, which were carefully measured by transmission electron microscopy (TEM). The grids were then rinsed by dipping three times in phosphate-buffered saline (without bovine serum albumin) and the cells were subsequently fixed by flotation on a drop of 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for 30 min. This was followed by either a brief rinse in buffer, or by storing the grid in buffer supplemented with 7% sucrose, in the refrigerator overnight. After a brief dehydration in 70, 90, and 100% ethanol the grids were dried at the critical point of CO_2, following routine procedures (16). Finally, they were mounted on modified aluminum stubs and coated by evaporation of ~10-15 nm of carbon, while affixed to a rotary-tilting stage (Ladd Research Industries, Inc., Burlington, VT; Cat. No. 60825). The specimens were kept in a vacuum desiccator until examined with the SEM. For TEM, similar prefixed cells were exposed to three comparable incubations while in cell suspension. They were finally pelleted in conical BEEM capsules, fixed with 2.5% glutaraldehyde and osmium tetroxide, ethanol-dehydrated, and embedded in an Epon/Araldite mixture.

SEM: For observation in the SEI mode the untilted specimen was examined at an accelerating voltage of 20 kV (Fig. 1). For observation in the backscattered electron imaging (BEI) mode, also at 20 kV, the reversed signal polarity was used (Fig. 2). SEI/BEI signals were then mixed, using the normal polarity of the BEI signal (Fig. 3). Although a JEOL-35 scanning electron microscope, equipped with a tungsten hair-pin cathode was used in the preliminary observations, a JEOL JSM 840 instrument, equipped with a lanthanum-hexaboride cathode, was used in the most recent studies as illustrated in this paper. Both microscopes were fitted with a similar solid-state BEI detector located immediately under the last lens pole piece and made up of an annular type high-sensitivity Si-P-N junction. All micrographs were taken on Polaroid P/N 55 films, at direct magnifications ranging from 10,000 to × 50,000.

RESULTS

The results obtained in the study of human peripheral granulocytes, labeled with a 45- or a 20-nm colloidal gold marker, after mild prefixation, are illustrated in Figs. 1-5. One granulocyte and two erythrocytes, visualized in the SEI mode of the SEM are seen in Fig. 1. This primarily topographical image illustrates the surface structures of a granulocyte, as typified mostly by broad surface ridges. The surface shows, however, a few particles distributed in the upper part of the cell. They presumably represent the 45-nm colloidal gold particles, labeling the granulocytic cell surface antigen against which the primary antibody has been specifically raised. Such particles were observed on the surface of all the granulocytes recognized in this preparation. They were conspicuously absent from the surface of the erythrocytes present in this cell sample. Higher magnification is obviously needed for better recognition of the gold marker in the SEI mode. However, occasional structures of marker size were observed on the surface of cells that were not expected to be labeled and made

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[2] D2 is a mouse IgM monoclonal antibody to normal human granulocytes (B. P. Gallagher and K. H. Shumak, manuscript in preparation). Culture supernatant containing D2 was a generous gift from Dr. K. H. Shumak, University of Toronto, Ontario.

us somewhat uncertain about the degree of specificity of the labeling.

This difficulty disappeared as soon as *the same cells* were observed in the BEI mode (Fig. 2). The contrast now being dependent on the atomic number, the gold marker (Au; Z = 79) is recognized in high contrast, although cell surface structures are no longer seen. The number of particles seen in Fig. 2 is considerably higher than that seen in the SEI mode. This difference in particle number is interpreted to reflect (a) the structural complexity of the cell surface, which, in a topographical image (Fig. 1), allows for the "hiding" of many particles behind microvilli or surface ruffles, and (b) the poor topographical contrast of a 45-nm particle embedded in a 10-15 nm carbon film. The gold particles are well-visualized in the BEI mode (Fig. 2) because the 20 kV electron beam penetrates most surface structures and because the image results from atomic number contrast. Control with the TEM was necessary (a) to exclude the possibility that, in spite of the mild prefixation, some of the particles observed in the BEI mode might have been internalized by endocytosis, and (b) to identify unambiguously the type of the labeled cell. As seen in Fig. 4, such control experiments clearly indicate that (a) the labeled cells are typical polymorphonuclear granulocytes; (b) the gold label is restricted to the cell surface; and (c) the surface of lymphocytes is predominantly unlabeled.

Cell surface labeling with the SEM is useful only insofar as direct correlation can be established between the presence of certain specific molecules exposed on cell surfaces (antigen, receptor sites, etc.) and the surface structures of these same cells. In Fig. 2, all surface structures are missing, and the image is, therefore, of limited interest per se.

In Fig. 3, however, the same portion of the same cells are illustrated by the superimposition of both the SEI and the SEI signals (signal mixing [17]). The polarity of the backscattered signal was set back to "normal" (therefore, gold particles are now white) and the SEI and BEI signals were mixed. The result is an image in which the distribution of all the gold particles can be precisely correlated with details of the surface structure of the cell.

It is realized that SEI of higher quality than that of Fig. 1 would have resulted from coating the cells with gold instead of carbon. Unfortunately, this would have completely masked the BEI signal of the gold marker. Granulocytes, similarly prefixed and primed with the monoclonal antibody D2 were also labeled with a 20-nm colloidal gold conjugate. As seen in Fig. 4, what appears to represent primarily single 20-nm gold particles can be resolved in the BEI mode and their distribution on cell surface efficiently correlated with surface structures, when viewed after mixing the BEI and the SEI signals. Note that the number of gold particles per surface area is markedly higher in Fig. 4 than in Fig. 3. This is interpreted as reflecting the decreased steric hindrance phenomena when a marker of smaller size is used (8), and a different ratio between the number of protein A molecules and the number of gold particles. The control for TEM

FIGURES 1-3 Fig. 1: One granulocyte and two erythrocytes labeled with 45 nm colloidal gold after incubation of the prefixed cells with an anti-mature granulocyte monoclonal antibody (D2). Viewed in the SEI mode, only a few gold particles are recognized in the upper portion of the surface of the granulocyte. Fig. 2: Same sample, same field as in Fig. 1, viewed in the BEI mode (reverse polarity). All the gold particles appear in good contrast over the entire surface of the cell, but the surface structures are no longer discernible. Fig. 3: Same sample, same field as in Fig. 1 and 2, viewed after mixing the SEI and the BEI (normal polarity) signals. The gold particles now appear white and are superimposed to the image of the cell surface.
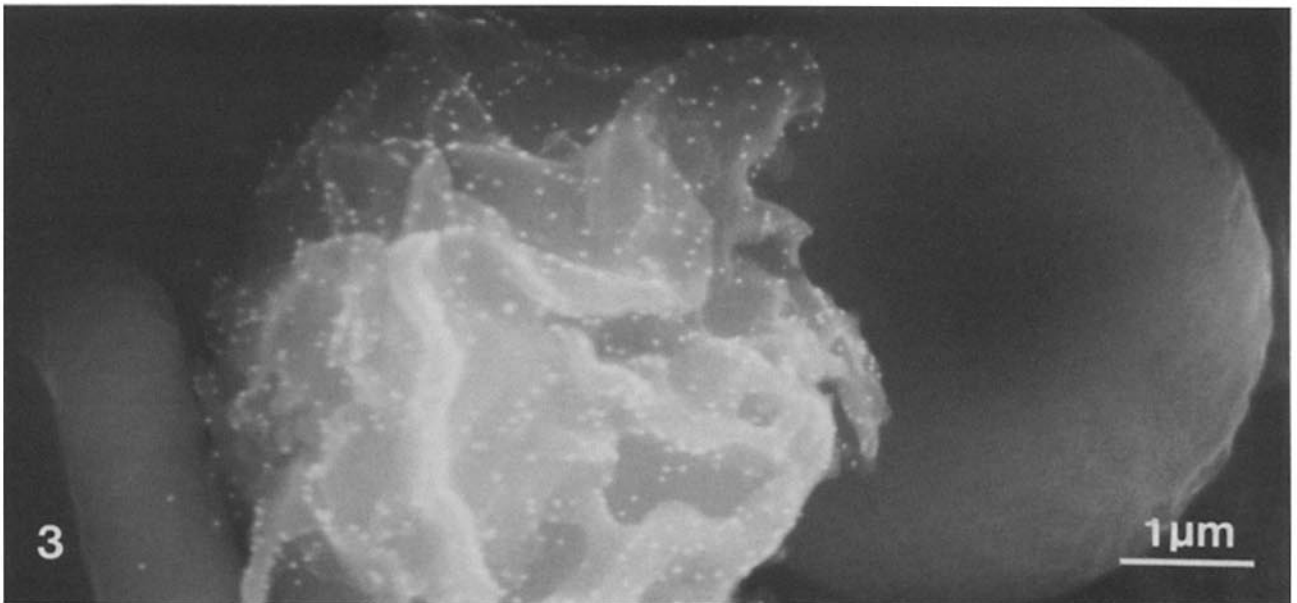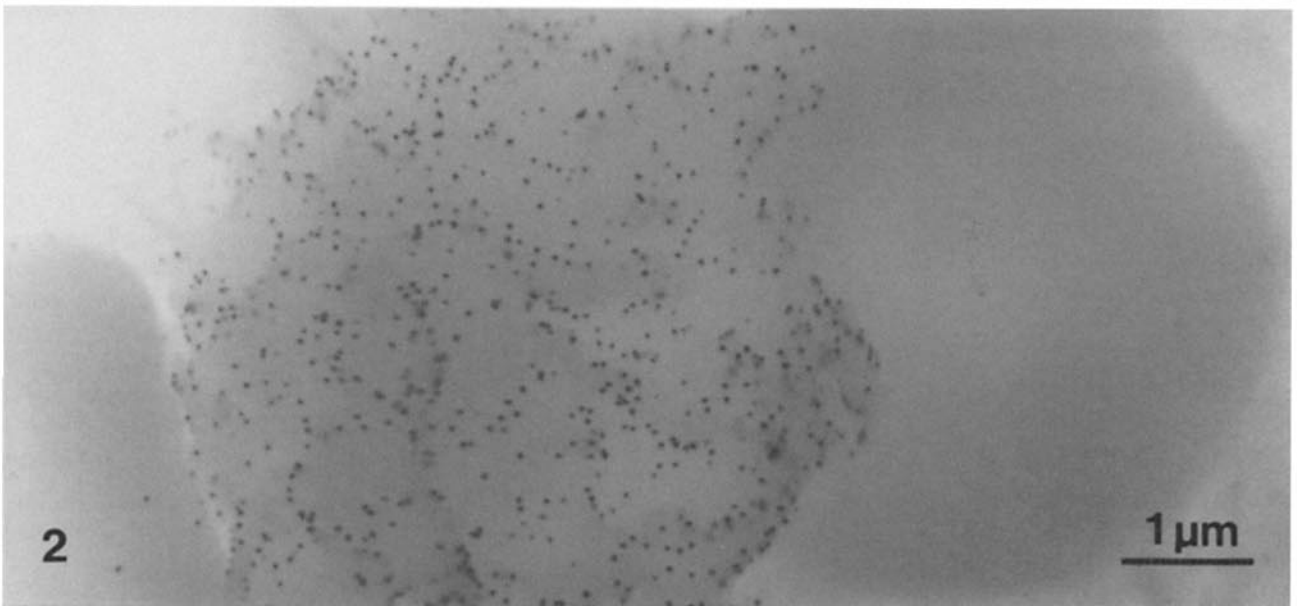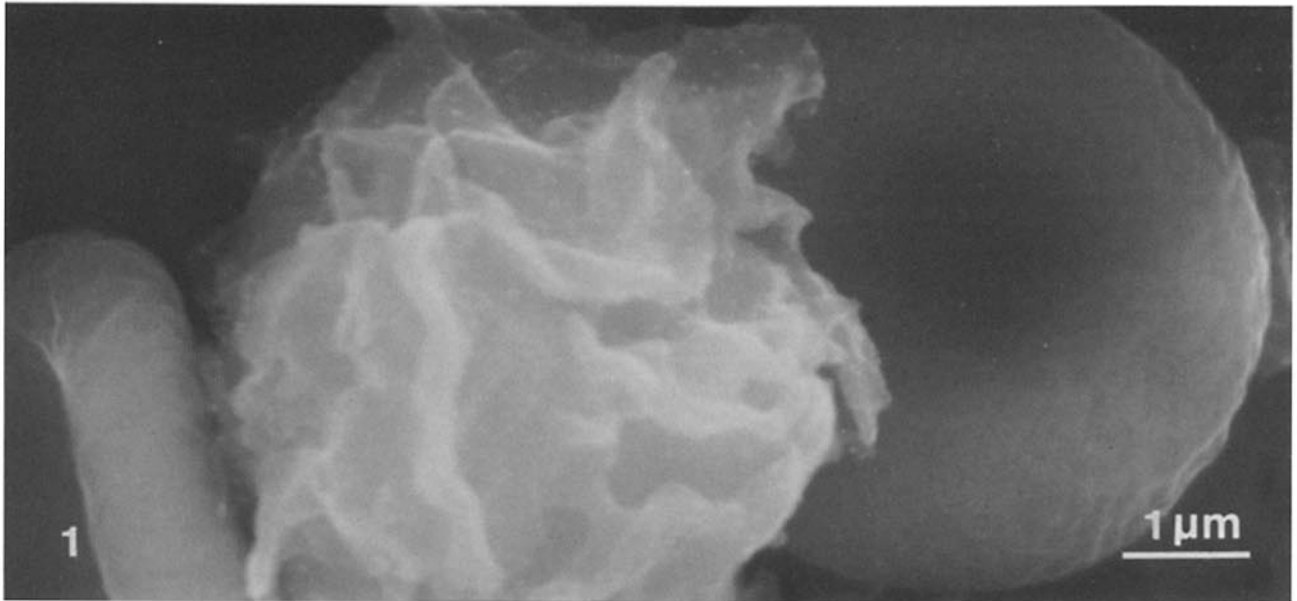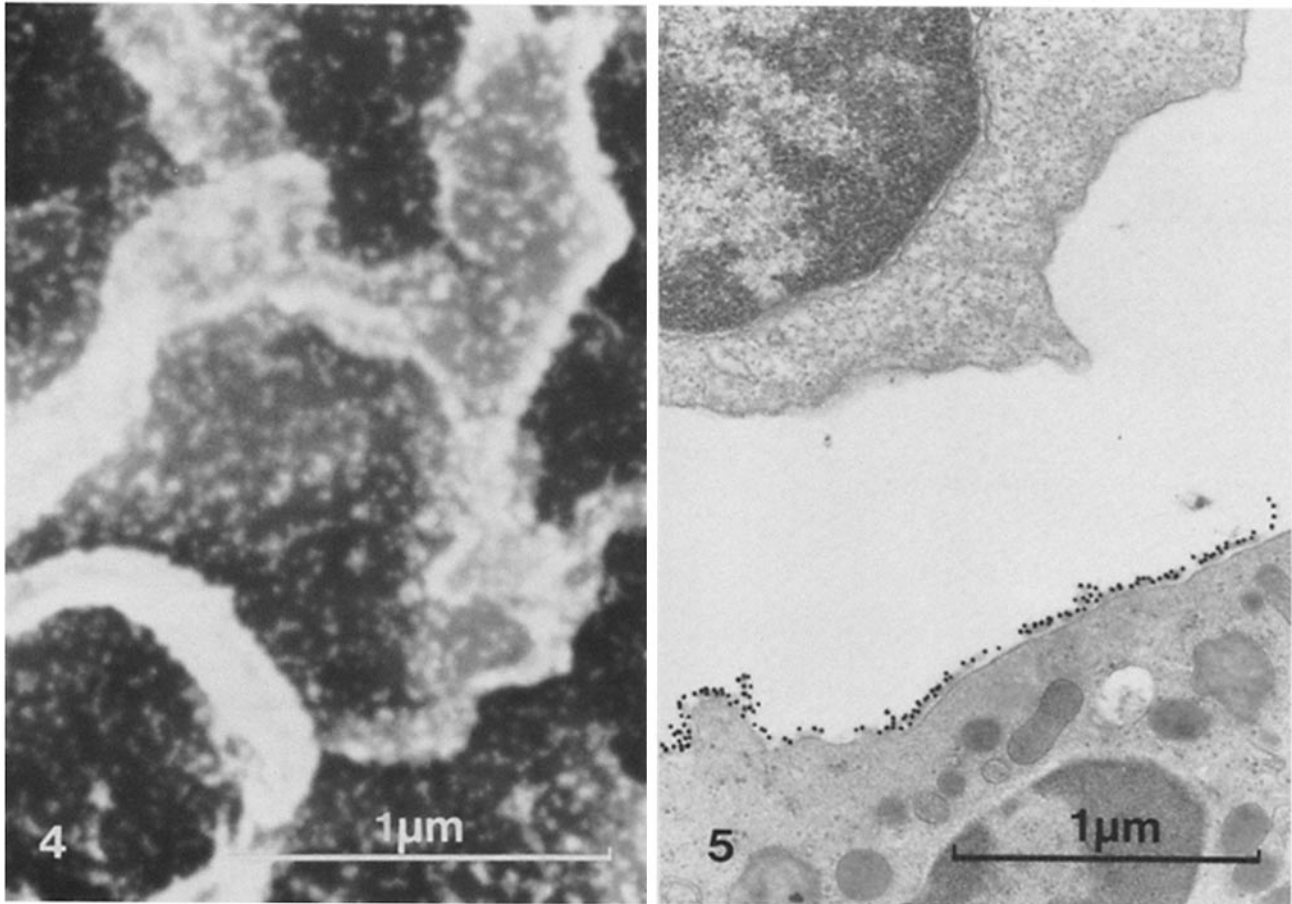
FIGURES 4 and 5 Fig. 4: A mixed SEI and BEI image of part of the surface of another granulocyte labeled as in Fig. 1, except that the 20-nm gold marker has been used. Note the good resolution of the 20-nm gold particles, and the density of the labeling with the 20-nm colloidal gold, which is much higher than that obtained with the 45-nm marker. Fig. 5: A TEM control of the experiment illustrated in Fig. 4. The specificity of the method is well illustrated by the intense surface labeling of the granulocyte and the absence of labeling of one lymphocyte.

illustrated in Fig. 5 was also prepared with the 20-nm gold marker. It similarly shows a labeling density markedly higher than what was observed in previous TEM experiments (not illustrated here) in which the 45-nm gold particles were used.

DISCUSSION

The method described here appears superior to the procedures used so far for the labeling of cell surfaces viewed with SEM. Four arguments supporting this statement will be briefly discussed.

(a) A marker of relatively small size (20 nm, plus an IgG layer surrounding the gold core) can be used, therefore minimizing the effects of steric hindrance phenomena (8), i.e., increasing the efficiency of the labeling procedure. Small markers have been used in the past in immuno-SEM studies (as small as ferritin, see reference 4). However, as long as conductive coating is needed to prevent the electrostatic charging of the cells, small markers in the 10–20-nm range become somewhat difficult to resolve in the SEI mode, "snowed in" under a conductive coating of ~10–20 nm in thickness.

(b) The atomic number contrast of gold particles is well-recognized when the surface of carbon-coated cells is observed in the BEI mode. As a result, all the particles attached to the exposed cell surface are apparently visualized. In the images

of the same area of the specimen observed in the SEI mode, only a small number of particles are visualized, presumably because of the masking effect of some surface structures or because some particles are exposed to the electron beam at an unfavorable incidence angle. We do not believe that the higher numbers of gold particles seen in the BEI mode result from viewing particles localized on the underside of the cells. This can probably be expected with cells thinly spread on the substrate. However, the cells studied here are more or less spherical in shape and have an average diameter frequently exceeding 5 or 6 μm. We know, from our previous work on the cytochemistry of blood cells viewed in the BEI mode (18), that a 20 kV electron beam does not penetrate cells to such a depth. Current studies, in which different accelerating voltages as well as stereo-pair imaging will be used, are expected to add further support to this statement. It already appears, however, that the precise quantitation of all labeled cell surface sites will become a practical possibility with the method described here. At variance, the SEI mode reveals only some of the gold particles and is therefore not appropriate for quantitative studies.

(c) Ascertaining the specificity of a labeling procedure with the SEM in the SEI mode is not easy because cells expected to be negative for labeling will occasionally carry on their surface contaminating particles of unknown origin that are, unfortunately, of marker size. In an imaging mode limited to

topography, these particles can be difficult to distinguish from the gold marker. Markers of characteristic shape, like haemocyanin (3) or bacteriophage T-4 (19), have been recommended in recent years to alleviate this difficulty. However, as soon as the identification of a marker is based on atomic number contrast, the problem is solved since it is very unlikely that the contaminating micro-debris would contain high atomic number elements, thus generating a BEI signal of comparable intensity to that given by the gold particles. In our current studies, we have encountered many SEI images in which rare particles were considered "suspect/positive," while these same particles were readily distinguishable from colloidal gold in the corresponding BEI image.

(d) Mixing the SEI and BEI signals has been previously recommended (17) and is a possibility offered on many commercially available scanning electron microscopes. In the method described here signal mixing is essential if one wishes to recognize the marker at fairly high resolution without losing the image of the accompanying cell surface structures. Direct correlations between the distribution of apparently all the labeled sites and the surface architecture of the cells can therefore be made.

One should emphasize that the choice of an optimum method will probably always depend on the nature of the different experimental materials. Methods including a mild and brief prefixation will be recommended for the labeling of cells in which clustering of the labeled surface sites is anticipated. This primarily results from the fact that cells prepared for SEM will have to be processed at room temperature, in view of the known damaging effects of low temperature, like +4°C, on cell surface architecture (20). However, for quantitative studies it will still be necessary to evaluate how much the activity of the antigen has been denatured by the "mild" glutaraldehyde prefixation. In other studies, currently progressing in our laboratory, subpopulations of T-derived human lymphocytes were also labeled with colloidal gold. In these experiments prefixation was not used, i.e., live cells were incubated with antibodies at room temperature. A certain level of clustering of the labeled sites as well as some endocytosis of the gold marker could not be satisfactorily avoided, in spite of the relatively high concentration of sodium azide used in these experiments. It was primarily to eliminate the interference of clustering and endocytosis that we followed the recommendations recently made (10, 21) and used the method illustrated here, which includes mild prefixation.

The novel approach to cell surface labeling recommended in this paper is characterized by its high efficiency and by the possibility to evaluate the number and distribution of the labeled sites. As such, it is expected to be of definite value in many studies related to the topographical distribution of various macro-molecules exposed on cell surfaces.

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