Scanning Microscopy

Volume 1 | Number 2 Article 27

2-16-1987

Immunogold Labeling of Human Leukocytes for Scanning Electron Microscopy and Light Microscopy: Quantitative Aspects of the Methodology

E. de Harven
University of Toronto

D. Soligo
University of Milan

Scanning Microscopy, Vol. 1, No. 2, 1987 (Pages 713-718)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0891-7035/87$3.00+.00

IMMUNOGOLD LABELING OF HUMAN LEUKOCYTES FOR SCANNING ELECTRON MICROSCOPY AND LIGHT MICROSCOPY: QUANTITATIVE ASPECTS OF THE METHODOLOGY

E. de Harven* and D. Soligo

*Department of Pathology, University of Toronto, Toronto, Ontario
Clinical Medical I, University of Milan, Milan, Italy

(Received for publication May 4, 1986, and in revised form February 16, 1987)

Abstract

When cell surface antigens are labeled with the colloidal gold marker, backscattered electron images (BEI) reveal all the gold particles and, therefore, permit total counts. Secondary electron images (SEI) show only a small percentage of the gold particles and are inadequate for quantitative evaluation.

For determination of the cellular labeling index, a time-consuming method implies the screening of 100 cells by scanning electron microscopy, at a magnification of approximately 12,000 to 15,000x, with continuous SE/BE shifts. A much more efficient method is to transfer the SEM sample or its equivalent under the light microscope and to count the total number of gold labeled cells in the epi-polarization mode. The total cell count can be evaluated under UV light, taking advantage of the autofluorescence of the glutaraldehyde fixed cells.

KEY WORDS: Immunogold labeling, leukocytes, quantitation, epi-polarization light microscopy, backscattered electron imaging.

*Address for correspondence:
Etienne de Harven
Department of Pathology
Banting Institute
100 College Street
Toronto, Ontario
Canada M5G 1L5 Phone No. (416) 978-2549

Introduction

The surface morphology of peripheral blood leukocytes (PBL's) is best studied by observing cells positively and individually identified with appropriate surface markers. Among all the markers which one can use in such studies (10), particles of colloidal gold, ranging in size from 5 to 40 nm, are most advantageous primarily because they can be seen with the light microscope, the transmission EM and the scanning EM as well. The colloidal gold marker can be used for the cytochemical labeling of surface glycoproteins, appropriately chosen lectins serving as identifier and ligand (7). It can also be used for the specific labeling of surface exposed antigens, various immunoglobulins acting as identifier and/or ligand in this case (12). We have been actively pursuing this second alternative, our choice being based on 1) the large number of available monoclonal antibodies (MoAbs), and 2) the capacity of many of these MoAbs to still identify their target antigens in spite of a mild prefixation with glutaraldehyde (11).

Colloidal gold particles were clearly demonstrated in SEM of cell surfaces by Horisberger (8). These observations were made in the secondary electron (SE) mode. An alternate imaging mode was illustrated by Trejdosiewicz (13), i.e., the backscattered electron (BE) mode. However, it was only in 1984 that BE imaging of colloidal gold particles acquired its full significance in quantitative studies. Indeed, one of us (de H.) demonstrated at that time that the number of colloidal gold particles counted on cell surface in the BE mode is considerably higher than that observed in the SE image of the same cell (1). Obviously, any attempt to quantitate the number of gold labeled epitopes exposed on cell surfaces should be based on the study of BE, not of SE images (3,4).

Methodology

Our immunolabeling method for human leukocytes, from healthy donors, has been described in detail elsewhere (3). Briefly, the Ficoll-Hypaque separated cells are first allowed to attach on a poly-l-lysine pretreated glass coverslip, then are prefixed with 0.2% glutaraldehyde for 10 min. A first incubation with diluted monoclonal antibody is followed by a second incubation with a goat anti-murine Ig/colloidal gold complex (GAM-G40, from Janssen Pharmaceutica, Beerse, Belgium) (5). Postfixation with 2.5% glutaraldehyde (1 h), ethanol dehydration, critical point drying from CO_2, and conductive coating with carbon only precede mounting on aluminum stubs and viewing with the JEOL JSM 840, equipped with a lanthanum hexaboride (LaB_6) cathode. Note that the conductive coating with carbon will of course limit the imaging of the gold marker in the SE but not in the BE mode of the SEM. Note also that the choice of monoclonal antibody is restricted to those monoclonals which can still recognize their target antigen after glutaraldehyde prefixation. An up to date list of such antibodies is presented in another paper (11). Glutaraldehyde prefixation is an essential part of the method because it totally prevents redistribution and endocytosis of the marker and because it insures that the incubated cells will retain a well preserved surface morphology.

The study of immunogold labeled cell samples with the SEM is carried out in three different modes: the SE, the BE with reverse polarity, and the mixed SE and BE images both in normal polarity. Comparative illustrations of the three imaging modes have been published previously (2,3).

Quantitative evaluation of gold surface labeling

Two different quantitative questions are considered: 1) how many gold particles on a given cell, and 2) what percentage of gold labeled cells in a mixed cell population (cellular labeling index)?

1. For direct counting, only BE images will be used (4) which will provide adequate information for counting on the original micrographs or on digitized images by computerized image analysis. Since steric hindrance restricts labeling efficiency (9), comparisons will only be made between samples labeled with the same size gold particles, for example 30 or 20 nm. With the probe current used (for example, 6×10^{-10} A) the resolution of the SE image is far from optimum. It permits, however, to correlate the precise localizations of the gold label with the surface features of the cell in mixed BE/SE images. Many factors have to be assessed to evaluate the possible biological significance of direct gold particle counts. These factors are reviewed in another publication (4).

2. For cellular labeling index determination, two different methods can be recommended, one based on SEM/BE, the other on epi-polarization light microscopy of the SEM samples. For direct SEM counting, one hundred cells will be sequentially viewed in the SE mode, at a standardized magnification (for example, 13,000x), minimizing subjective bias in selecting the cells. Each cell will be focussed in the SE mode and then briefly viewed in the BE mode to assess gold labeling. A threshold of non specific background will be arbitrarily set (at 5 gold particles per cell in our practice). Sixty five percent positively labeled cells were counted by this method in the case of human T-cells (Fig. 1) identified with the Leu-1 monoclonal antibody (Becton Dickinson). B cells identified with the B1 monoclonal antibody (Coulter), also labeled with GAM-G40, gave a cellular labeling index averaging only 11.8%.

An alternate approach, faster but less accurate, is carried out at low magnification (1600x), many cells being viewed in a single field. At this magnification, however, the individual gold particles are not resolved and a precise categorization between labeled and unlabeled cells becomes ambiguous.

Far more satisfactory for cellular labeling index determination is the transfer of the SEM sample, mounted on its stub, to the stage of the light microscope, for epi-polarization microscopy (EPM), according to DeMey (5,6). A Cambridge stub can be mounted on a 7.5 x 2.5 cm plastic plate, and positioned on the stage of the light microscope (Fig. 2) We use the Nikon Microphot-FX, with the IGS block for epi-polarization microscopy and with a 60X plan-apochromatic oil immersion lens. This gave adequate visualization of the labeled cells (Fig. 3) which can be accurately counted. Unlabeled cells are difficult to see in the epi-polarization mode, and our opaque cell substrate (i.e., the SEM stub!) does not permit of course to combine trans-illumination. Fortunately, autofluorescence of the glutaraldehyde fixed cells permits one to quickly obtain a total cell count of any UV illuminated field of view (Fig. 4), and therefore to calculate the exact labeling index. To avoid sacrificing the SEM sample for EPM observation, one can, alternatively, prepare two identical cell carrying

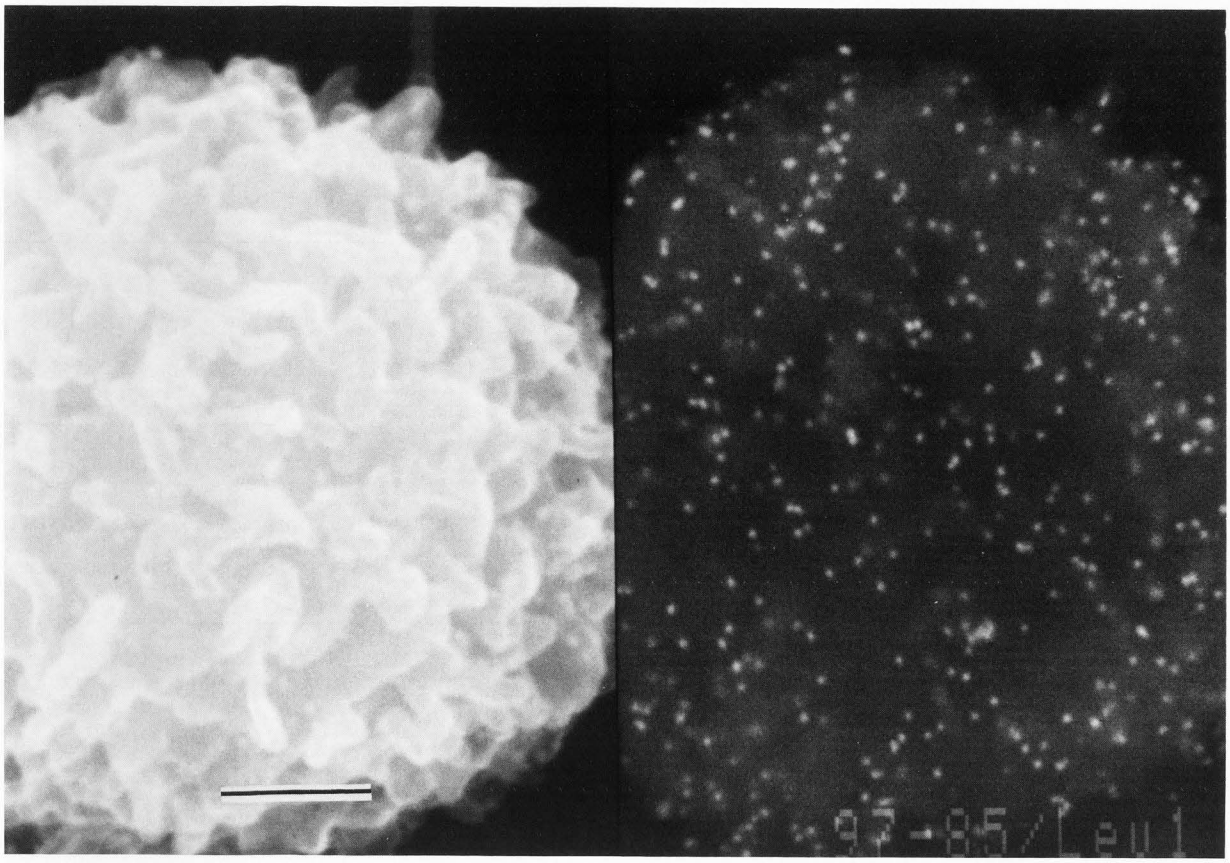

Figure 1. A multiple display image of one Leu-1 +ve T lymphocyte viewed simultaneously in the SE (left) and the BE (right) modes. The number of colloidal gold particles seen in the SE image is considerably smaller than that in the BE image (bar = 1 μm).

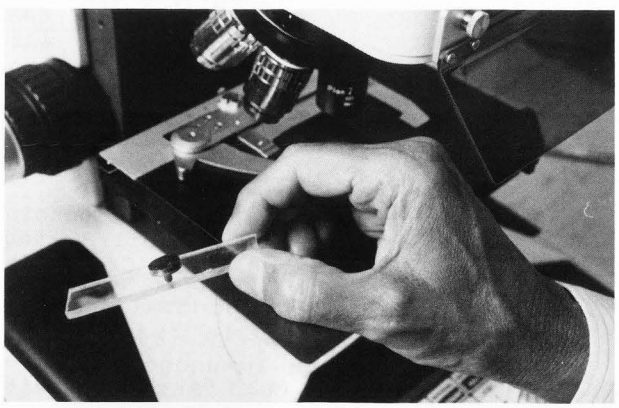

Figure 2. The SEM sample is mounted on a plexiglass slide for direct transfer to the stage of the light microscope and observation first in the epi-polarization mode and finally in epi-fluorescence.

glass coverslips and mount one on a stub for SEM and the other on a glass slide for EPM study. The well known phenomenon of autofluorescence after glutaraldehyde fixation is usually regarded as a nuisance in many experiments. In our application, however, it considerably facilitates total cell count, eliminating the need to use stain or other methods of contrast enhancement.

Conclusions

The colloidal gold marker for the labeling of surface antigens of glutaraldehyde prefixed cells represent a unique tool for quantitative studies provided the SEM observations are made in the backscattered electron (BE) imaging mode, and provided cellular labeling index determinations as well as all necessary control observations are carried out in epi-polarization light microscopy on the very same (or equivalent) cell samples as those prepared for SEM/BE.

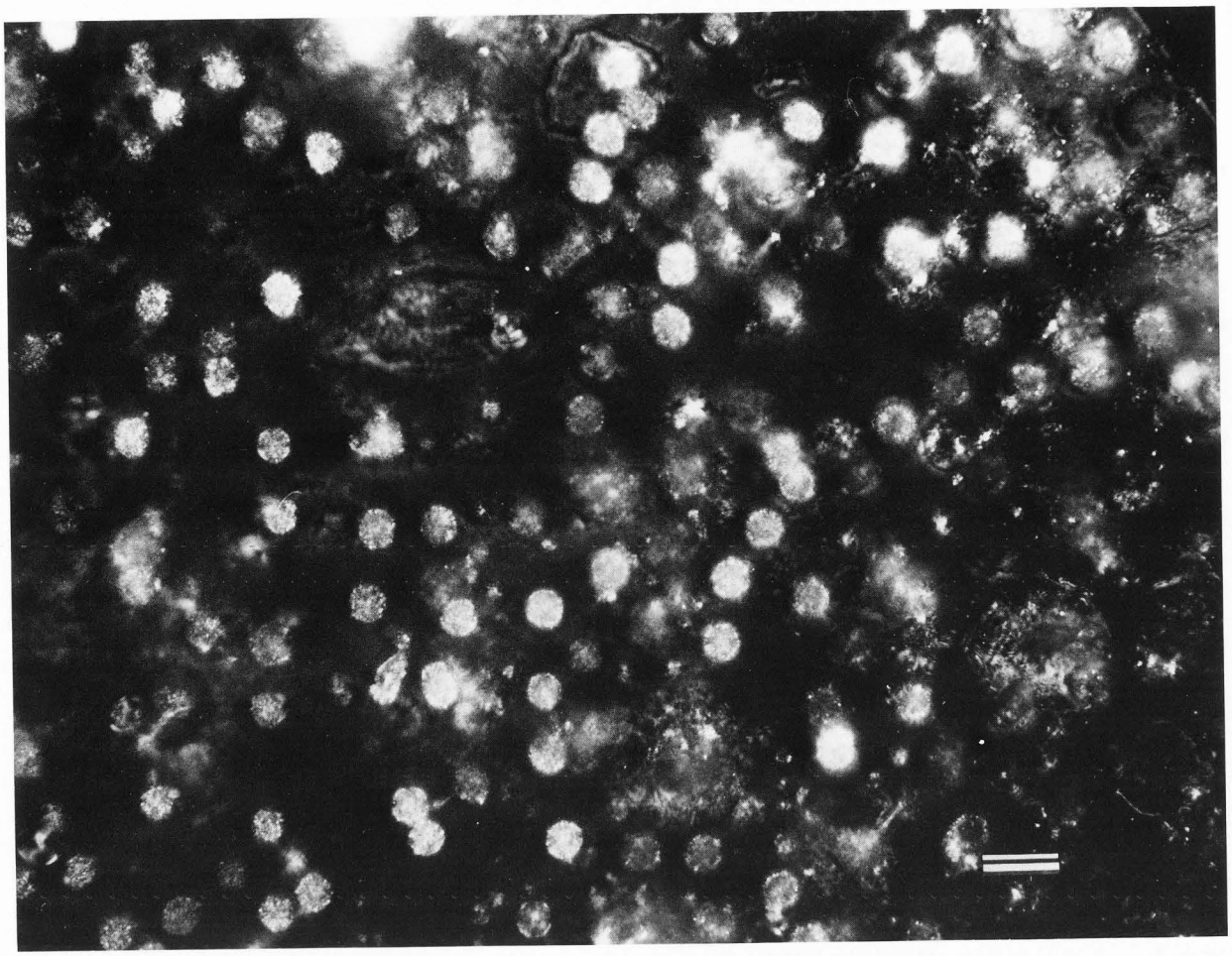

Figure 3. The epi-polarization mode clearly reveals the total number of gold labeled lymphocyte in this SEM sample (bar = 10 um).

Acknowledgments

The assistance of Mrs. H. Christensen in preparing all the samples used in this study, and of Mr. G. Reschauer, Nikon Canada, Inc., in setting up the epi-polarization microscope in our laboratory is greatly appreciated. The assistance of Miss L. Bortolamiol in preparing the typescript is also gratefully acknowledged.

Supported in part by grants from the Medical Research Council of Canada and of the Leukemia Research Fund (Toronto) (E. de Harven) and from the Italian National Research Council (to D. Soligo).

References

1. de Harven E, Leung R, Christensen H. (1984) A Novel Approach for Scanning Electron Microscopy of Colloidal Gold-labeled Cell Surfaces. J. Cell Biol. 99, 53-57.

2. de Harven E, Soligo D. (1985) Backscattered Electron Imaging of Colloidal Gold Markers for Leukocyte Surface Antigens. Proceedings of the 43rd Annual Meeting of the Electron Microscopy Society of America, (San Francisco Press, Inc., Box 6800, San Francisco, CA 941016800, USA,) 534-537.

3. de Harven E, Soligo D. (1986) Scanning Electron Microscopy of Cell Surface Antigens Labeled with Colloidal Gold. Am. J. of Anat. 175, 277-287.

4. de Harven E, Soligo D. (1987) Should We Be Counting Immunogold Marker Particles on Cell Surfaces with the SEM? J. Microsc. (In press).

5. De Mey J, Moeremans M, Geuens G, Nuydens R, De Brabander M. (1981) High resolution light and electron microscopic localization of tubulin with the IGS (immuno-gold staining) method. Cell Biol. Int. Rep. 5:889-899.

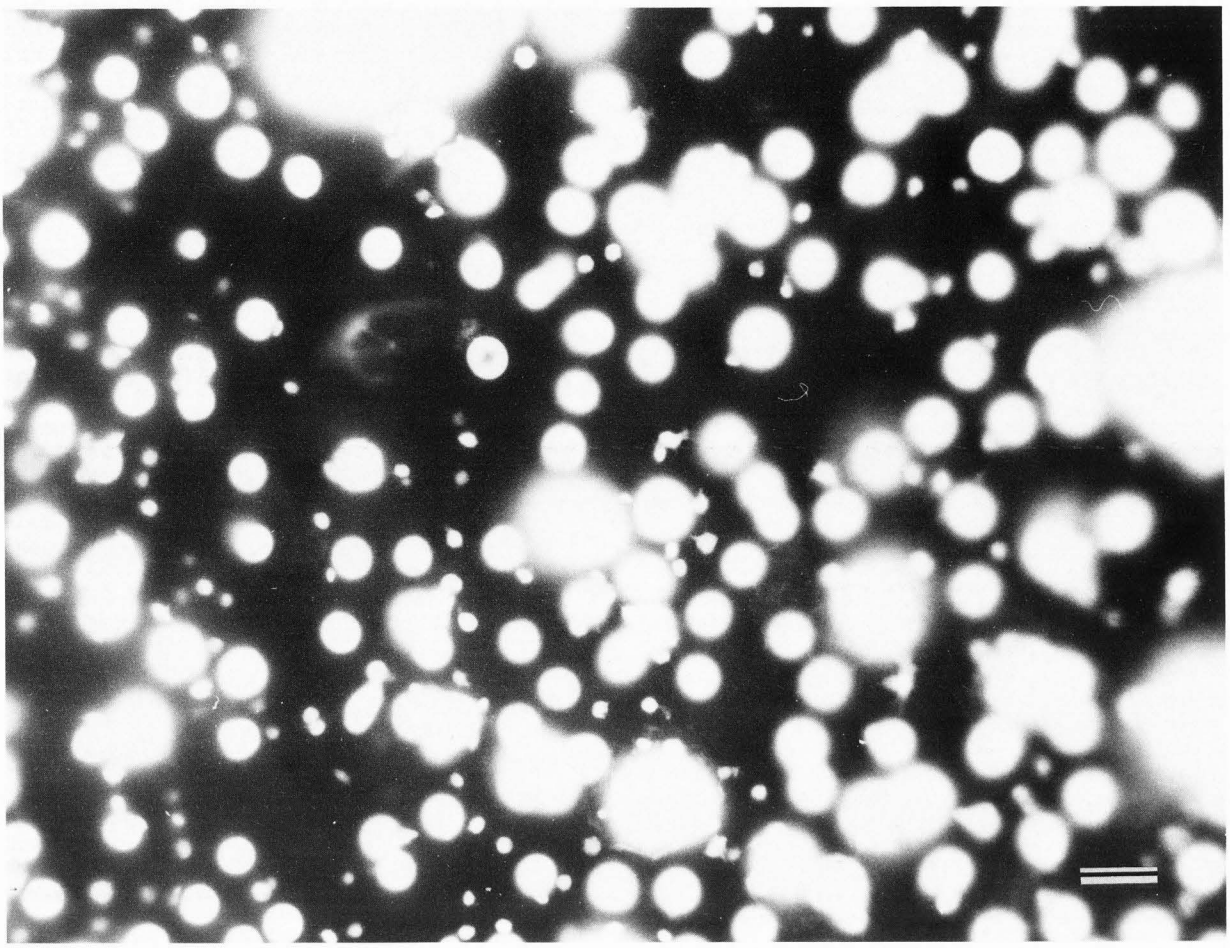

Figure 4. The same field is briefly epi-illuminated with UV light for demonstration of the auto-fluorescence of all these glutaraldehyde fixed cells. A total lymphocyte count is easy on such pictures (eliminating, of course, the small platelets and the large, spread out monocytes) which one has to take quickly before the auto-fluorescence fades away (bar = 10 μm).

6. De Mey J, Springall D, De Waele M, and Hacker G. (1986) Gold probes in light microscopy. In: Immunocytochemistry. Modern methods and applications. J. M. Polak and S. Van Noorden, (Eds.), 2nd edition. Wright, PSG, 71-88.

7. Horisberger M. Rosset J. (1976) Localization of wheat germ agglutinin receptor sites on yeast cells by scanning electron microscopy. Experientia 32, 998-1000.

8. Horisberger M. (1979) Evaluation of Colloidal Gold as a Cytochemical Marker for Transmission and Scanning Electron Microscopy. Biol. Cellulaire 36, 253-258.

9. Horisberger M, Tacchini-Vonlanthen M. (1983) Stability and Steric hindrance of lectin-labeled gold markers in transmission and scanning electron microscopy. Lectins, vol. III T.C. Bog-Hansen, G.A. Spengler (Eds,), Walter de Gruyter & Co., Berlin, Germany, 189-198.

10. Molday RS, Maher P. (1980) A review of cell surface markers and labeling techniques for scanning electron microscopy. Histochem. J. 12, 273-315.

11. Soligo D, Lembertenghi-Deiliers G, de Harven D. (1987) Immunoscanning Electron Microscopy of Normal and Leukemic Labeled with Colloidal Gold. Scanning Microscopy 1 (2), 719-725.

12. Soligo D, de Harven E, Nava MT, and Lambertenghi-Deliliers G. (1986) Immuno-Cytochemistry with Backscattered Electrons. In: Science of Biological Specimen Preparation, 1985, Proc. of the 4th Pfefferkorn Conference, M. Mueller et al., (eds.), SEM, Inc., AMF O'Hare, IL, 289-297.

13. Trejdosiewicz LK, Smolira MA, Hodges GM, Goodman SL, Livingston DC. (1981) Cell surface distribution of fibronectin in cultures of fibroblasts and bladder derived epithelium: SEM-immunogold localization compared to immunoperoxidase and immunofluorescence. J. Microsc. 123:227-236.

Discussion with Reviewers

G.M. Hodges: For labeling index determination the resolving of individual gold particles on labeled cells would not seem essential. Therefore, in that even at low magnification BEI allows gold labeling to be seen in good contrast on the surface of specimens, is there not a case for developing further this approach which obviates the problem of autofluorescence fading and possible inaccuracies in total cell counts encountered in the LM total cell counting methodology described in this paper?
Authors: The answer is yes, of course. Unfortunately, in samples with a relatively weak labeling intensity we have found it difficult to unambiguously classify each cell as +ve or -ve, and we prefer, therefore to take the time to visualize gold particles on each positively labeled cell. In addition, we could not use an arbitrary level of non-specific background of 5 particles per cell without resolving them at relatively high magnification.

G.M. Hodges: You emphasize that glutaraldehyde prefixation is an essential part of your methodology for the immunogold labeling of human leukocytes for SEM. a) What problems have you encountered with paraformaldehyde or paraformaldehyde-glutaraldehyde prefixation schedules as used in the literature: is it your experience that such prefixation are less than adequate for leukocyte studies? b) What evidence have you that 0.2% glutaraldehyde prefixation for 10 min totally prevents redistribution and endocytosis of the marker?
Authors: a) Since this paper was submitted for publication we have obtained excellent results with 3% paraformaldehyde supplemented with 0.1% glutaraldehyde. However, surface preservation was not satisfactory when paraformaldehyde alone was used.
b) For redistribution, our evidence is based on the fact that we have never observed any clustering or patchy distribution of the gold labeled antigenic sites. For endocytosis, our evidence is based on several control samples (not reported in this paper) prepared for TEM by using the same prefixation step, i.e., 0.2% glutaraldehyde for 10 min, and in which we could never observe any gold particle within an endocytotic vesicle.

K.R. Peters: Does your "cellular labeling index" vary with the size of gold markers applied?
Authors: We have not yet systematically compared markers of different sizes under otherwise identical experimental conditions.

K.R. Peters: How great is the volume loss of the cells after your preparatory procedure, and does it affect SEM imaging and SEM-label density determination?
Authors: As well known for many years, critical point drying inevitably results in cell shrinkage. We made no experiment aimed at measuring this artefact. We hope to minimize it by using a more complex postfixation schedule with which work is currently in progress.

H. Gamliel: To my knowledge, no one has ever shown nor proved that mild fixation with 0.2% glutaraldehyde totally prevents redistribution and endocytosis of the marker. This sort of fixation can neither insure that the incubated cells will retain a well preserved surface morphology.
Authors: The first part of this question has already been answered (see answer to G.M. Hodges's question). As far as "insuring to retain a well preserved surface morphology", all I can tell you is that control samples, unincubated and unlabeled and prepared by the best proven methods of SEM hematology, were undistinguishable.

J. De Mey: Your use of the autofluorescence of the cells for total cell counting, when using epipolarization microscopy for differential counting is elegant. Are there problems with cell type recognition in mixtures of cells? How do you distinguish for example a monocyte from a lymphocyte?
Authors: This does not seem to be a major problem because, in this type of preparation, monocytes usually spread and can therefore be discounted on the basis of their larger size. I agree that, especially in hypercellular preparations, cell superimposition may represent an occasional problem.